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(54) *Bacillus thuringiensis* sporulation gene

(57) The present invention relates to a novel sporulation gene isolated from *B. thuringiensis*, and a DNA segment comprising the nucleic acid sequences encoding the gene and DNA sequences encoding crystal toxin proteins wherein said DNA segment is stably integrated into a *B. thuringiensis* host by homologous recombination.

The invention further relates to a DNA segment wherein the sporulation gene is mutated thereby rendering an oligosporogenic or asporogenic transformed *B. thuringiensis* as a result of stable integration of the DNA segment into the *Bacillus thuringiensis* chromosome by homologous recombination.

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Description

BACKGROUND OF THE INVENTION

It is well known that *Bacillus thuringiensis*, which accounts for the majority of all biopesticides used today, produces a crystalline inclusion during sporulation, and it is this crystalline inclusion which is composed of one or more δ -endotoxins and is responsible for toxicity to a wide range of insect hosts including larvae of lepidopteran, coleopteran, and dipteran insects.

Due to considerable interest in the use of *B. thuringiensis* as a biological pesticide, numerous studies have been done on identification of the insecticidal proteins and genes encoding these proteins in *B. thuringiensis* strains. However, less is known about the genes involved in the sporulation pathway. The sporulation genes are not only responsible for sporulation but also are associated with crystal production. Most insecticidal crystal proteins are expressed only during sporulation. Therefore, the identification and characterization of the genes involved in the sporulation pathway would enhance our knowledge and ability to manipulate crystal production and the sporulation cycle, and further would potentially increase the effectiveness of *B. thuringiensis* as a biological pesticide.

The production of viable spores by recombinant *B. thuringiensis* strains can be a disadvantage with respect to the use of *B. thuringiensis* products. All commercial *B. thuringiensis* strains form spores which are released into the surrounding environment in combination with the insecticidal proteins and while the spores may provide some insecticidal advantage, they are highly durable structures and can survive in the soil under extreme weather conditions.

The use of asporogenic or oligosporogenic *B. thuringiensis* in biopesticides could prevent or diminish the release of these viable spores making the use of *B. thuringiensis* biopesticides an even more attractive alternative to the use of conventional pesticides. Moreover, documentation in the literature suggests that over expression of certain *cry* genes can occur in asporogenic strains. For example, expression of the *cryIII A* gene has been observed to increase in sporulation mutants of *Bacillus* strains.

The morphological and physiological changes that occur during sporulation have been studied extensively in *Bacillus subtilis*. In general once sporulation is initiated, the cells undergo a number of morphological stages and sporulation involves a radical change of the biosynthetic activity of the bacterium. As sporulation begins, the chromosome condenses. At stage II, cell division occurs producing sister cells that are different in size. The smaller cell is the daughter cell, also known as the forespore or prespore. The larger cell is designated the mother cell. During stage III, the mother cell engulfs the forespore resulting in the formation of a double-membrane around the forespore inside the mother cell. A modified form of a cell wall known as cortex is synthesized between the inner and outer membranes of the prespore during stage IV followed by spore coat deposition on the outer membrane of the prespore during stage V. Stage VI is defined as the complete maturation of the spore. At this stage the spore develops its characteristic properties of resistance to radiation, heat, lysozyme, and organic solvents. Finally, the mother cell lyses and the mature spore is released in stage VII. The free spore is refractile and can be easily observed using light microscopy.

Genes that are needed for sporulation can be recognized by creating mutations which permit normal vegetative growth, but block sporulation. In *Bacillus subtilis* at least 100 sporulation genes have been identified which are involved in the sporulation process. The genes are designated as *spo0*, *spoII*, *spoIII*, etc. depending upon the stage in which sporulation is blocked. Genes involved in later events of sporulation in *Bacillus subtilis* have been identified as *spoV* genes and *spoV A*, *B*, *D*, *Ea*, *Eb*, *G*, *Id*, *J* and *R* have been identified in databases. The capital letters indicate loci containing mutations conferring similar phenotypes but mapping at distinct chromosomal positions. Sporulation and gene expression and control in *Bacillus subtilis* is further discussed in Errington, Jeffrey, *Bacillus subtilis* Sporulation: Regulation of Gene Expression and Control of Morphogenesis, Microbio. Rev. 57 (1-33), which is hereby incorporated by reference.

The present invention is the first known isolation of a stage V sporulation gene from a host *B. thuringiensis* strain. The present gene designated *spoVBt1* is about 65.5% homologous to the *B. subtilis* *spoVJ* gene at the nucleotide level and, the transposon, Tn917 was used as a tool for the identification of *spoVBt1*.

The novel *spoVBt1* gene and related *spoV* DNA sequences (as defined below) are used in a method of stably introducing exogenous DNA into bacteria. The *spoV* sequences of the invention are substantially homologous to a fragment of a sporulation gene located on a bacterial chromosome. The bacterial fragment comprising a sporulation gene serves as a site for chromosomal integration of the exogenous DNA and *spoV* sequence.

Surprisingly it has also been found that if the *spoVBt1* gene and other *spoV* DNA sequences are mutated by, for example, point mutations not only will exogenous DNA be incorporated into the bacterial chromosome but also the recipient bacteria will form mutated spores.

SUMMARY OF THE INVENTION

The present invention relates to an isolated *spoVBt1* gene having the nucleotide sequence as shown in SEQ. ID

NO.1. This invention further relates to an isolated spoV DNA sequence selected from the group consisting of i) the above-identified isolated *spoVBt1* gene; ii) a nucleotide sequence encoding a *Bacillus thuringiensis* sporulation protein as depicted in SEQ. ID NO.2; iii) a nucleotide sequence encoding a *Bacillus thuringiensis* sporulation protein substantially similar to the protein depicted in SEQ. ID NO.2; iv) a nucleotide sequence which hybridizes to a complementary strand of a sequence of i), ii) or iii), under stringent hybridization conditions and v) a truncated nucleotide sequence of i), ii), iii) or iv) above wherein said truncated sequence includes at least 300 nucleotides and more preferably at least 500 nucleotides.

The invention further relates to a DNA segment comprising the spoV DNA sequence defined above linked to a DNA sequence encoding at least one insecticidal crystal protein. The codons of said spoV DNA sequence comprise nucleotide sequences substantially homologous to sequences present in *Bacillus thuringiensis* chromosomal DNA and this allows for recombination. This DNA segment may be chromosomally integrated into a host *Bacillus thuringiensis*. The *B. thuringiensis* chromosomal fragment which is substantially homologous to the spoV DNA sequence serves as an integration site for the DNA segment. In this manner the invention includes an increase in the crystal gene content of a bacterium.

The invention also comprises a DNA segment comprising a mutated spoV DNA sequence (defined herein below) operably linked to a DNA sequence encoding at least one insecticidal crystal toxin protein wherein codons of said mutated spoV DNA sequence comprise nucleotide sequences substantially homologous to sporulation gene sequences present in *Bacillus thuringiensis* chromosomal DNA so that the DNA segment is capable of being inserted into the bacterial chromosomal sporulation gene locus and replicated and further the insecticidal crystal toxin protein is capable of being expressed in a *Bacillus* host wherein said host is rendered asporogenic or oligosporogenic.

The invention has particular relevance to recombinant *B. thuringiensis* strains wherein toxic crystal proteins are expressed by a transformed host but wherein spores are released into the environment. Therefore, in addition, the invention concerns a method of preparing asporogenic or oligosporogenic insecticidal crystal protein producing *Bacillus thuringiensis* strains comprising a) obtaining a DNA segment which includes a mutated spoV DNA sequence operably linked to at least one and no more than three insecticidal crystal protein encoding sequences; b) introducing said segment into a *Bacillus thuringiensis* host capable of sporulation; c) allowing homologous recombination to occur between the DNA segment and a substantially homologous nucleotide fragment of a sporulation gene in the host *Bacillus thuringiensis* chromosome wherein said DNA segment is stably integrated into the *Bacillus thuringiensis* chromosome and disrupts the sporulation process and; d) isolating a stably transformed asporogenic or oligosporogenic *Bacillus thuringiensis* host transformant wherein said stably transformed host is capable of expressing the introduced insecticidal crystal protein sequences.

A further object of the invention includes the transduction of a transformed *Bacillus thuringiensis* host comprising exposing the transformed host to a transducing phage; allowing said phage to replicate in said host wherein one to three exogenous insecticidal crystal protein encoding DNA sequences integrated into said *Bacillus thuringiensis* host chromosome are incorporated into the phage and introducing the insecticidal crystal protein encoding DNA sequence from said phage into a recipient *Bacillus thuringiensis* wherein said introduced exogenous crystal protein encoding DNA sequence is stably incorporated into said chromosome of the recipient and expressed in said recipient. The recipient *Bacillus thuringiensis* may or may not be rendered asporogenic or oligosporogenic depending on the DNA segment.

In this regard the invention includes a method of using a *Bacillus thuringiensis* chromosomal sporulation gene fragment as locus for chromosomal integration of a DNA segment, the DNA segment comprising at least one insecticidal crystal protein encoding gene, preferably one to three insecticidal crystal protein encoding genes, wherein said gene (s) is (are) stably integrated into the *Bacillus thuringiensis* chromosome.

The invention further relates to a broad spectrum insecticidal composition comprising an insecticidally effective amount of a transformed *B. thuringiensis* according to the invention and an acceptable carrier thereof.

Another objective of the present invention is the genetic engineering of a *B. thuringiensis* host whereby use of said host for the control of pathogenic insects provides an environmentally safer biopesticide wherein viable spores are not released into the environment.

A further object of the invention includes a method of protecting crop plants comprising applying to the locus where control is desired a composition of the invention.

Other aspects of the present invention will become apparent to those skilled in the art from the following description and figures.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 illustrates the predicted amino acid sequence of the isolated *spoVBt1* gene as illustrated in SEQ ID NO.1 and corresponds to SEQ. ID No.2.

FIGURE 2 illustrates the *spoVBt1* gene interrupted with Tn917 and the location of oligonucleotides used for sequencing.

qu ncing.

FIGURE 3 illustrates the plasmids, pSB901, pBR322 and pSB140.

FIGURE 4 illustrates plasmid pSB210.

FIGURE 5 illustrates plasmid pSB1207.

FIGURE 6 illustrates plasmid pSB1209.

FIGURE 7 illustrates plasmid pSB1218.

FIGURE 8 illustrates plasmid pSB1219.

FIGURE 9 illustrates plasmid pSB1220.

FIGURE 10 illustrates plasmid pSB139.

FIGURE 11 illustrates plasmid pSB210.1.

FIGURE 12 illustrates plasmid pSB32.

FIGURE 13 illustrates plasmid pSB219.

FIGURE 14 illustrates plasmid pSB458.

FIGURE 15 illustrates plasmid pSB1221.

DETAILED DESCRIPTION OF THE INVENTION

The isolation and purification of a sporulation gene from *B. thuringiensis* HD1Mit9::Tn917 is described at length in Example 1. The nucleotide sequence is shown in SEQ. ID NO. 1. The molecular weight of the putative protein product is calculated as 36.7 kDa. The sporulation gene is designated *spoVBt1*. The predicted amino acid sequence is shown under the nucleotide sequence. The putative ribosome binding site includes nucleotides 459 through 470 of SEQ ID NO.1 and the predicted transcription terminator stem-loop includes nucleotides 1415 through 1424 and 1431 through 1440.

The invention also includes those nucleotide sequences which encode the protein of SEQ. ID NO.2. It will be appreciated by those skilled in the art that an amino acid is frequently encoded by two or more codons, for example the amino acid leucine is encoded by the nucleic acid sequences of the following codons, TTA, TTG, CTT, CTA, CTG and CTC. Codons which code for the same amino acid are considered synonymous codons.

The invention still further embodies nucleotide sequences which encode sporulation proteins that are substantially similar to the protein depicted in SEQ. ID NO.2. The term substantially similar to the protein depicted in SEQ. ID NO. 2 means that the proteins are stage V sporulation proteins and the degree of similarity of the amino acid sequences is preferred to be at least 80%, more preferred the degree of similarity is at least 85%, and most preferred the degree of similarity is 95% to SEQ. ID No:2. A nucleotide sequence encoding a stage V sporulation gene includes those genes involved in late events of the sporulation process. For example those genes involved in deposition of spore coat protein, development of germination processes and progressive acquisition of resistance to organic solvent, heat and lysozyme to name a few.

In the context of the present invention, two amino acids sequences with at least 85% similarity to each other have at least 85% identical or conservatively replaced amino acid residues.

For the purpose of the present invention conservative replacements may be made between amino acids within the following groups: (i) alanine, serine and threonine; (ii) glutamic acid and aspartic acid; (iii) arginine and lysine; (iv) asparagine and glutamine; (v) isoleucine, leucine, valine and methionine; and (vi) phenylalanine, tyrosine and tryptophan.

The invention still further includes nucleic acid sequences which are complementary to one which hybridizes under stringent conditions with any of the above disclosed nucleic acid sequences. A first nucleotide sequence which "hybridizes under stringent hybridization conditions" to a second nucleotide sequence can not be substantially separated from the second sequence when the second sequence has been bound to a support and the first and second sequences have been incubated together at 65°C in 2x standard saline citrate containing 0.1% (w/v) sodium dodecyl sulphate, the thus hybridized sequences then being washed at 50°C with 0.5x standard saline citrate containing 0.1% (w/v) sodium dodecyl sulphate.

Specifically, the *spoVBt1* gene depicted in SEQ. ID NO.1 is a gene that is associated with later stages of the sporulation process. In this regard the nucleotide sequences of the invention are referred to under the general heading of *spoV* DNA sequences and more specifically are identified as i) a *spoVBt1* gene having the nucleotide sequence shown in SEQ. ID NO.1; ii) a nucleotide sequence encoding a *Bacillus thuringiensis* sporulation protein as depicted in SEQ. ID NO.2; iii) a nucleotide sequence encoding a *Bacillus thuringiensis* sporulation protein substantially similar to the protein depicted in SEQ. ID NO.2; iv) a nucleotide sequence which hybridizes to a complementary strand of i), ii) or iii) under stringent hybridization conditions; and v) a truncated nucleotide sequence of i), ii), iii) or iv) above wherein the truncated sequence includes at least 300 nucleotides and more preferably at least 500 nucleotides.

While a truncated *spoV* DNA sequence is most preferably at least 500 nucleotides, a particularly preferred sequence is base pair 488 to 1404, inclusive, of SEQ ID NO.1. This particular sequence is referred to herein as (I)

spoVBt1-1.

Accordingly, the invention provides, a DNA segment comprising a *spoV* DNA sequence as defined above or a mutated *spoV* DNA sequence as defined herein below operably linked to other DNA sequences wherein the DNA sequences encode exogenous or foreign proteins. For example in a preferred embodiment the DNA segment may include in addition to the *spoV* DNA sequence, insecticidal crystal protein encoding DNA sequences. Preferably, the DNA segment will include one to three insecticidal protein encoding genes. Such sequences include but are not limited to *cryIA(a)*, *cryIA(b)*, *crf1A(c)*, *cryIB*, *cryIC*, *cryIC(b)*, *cryID*, *cryIE*, *cryIF*, *cryIG*, *cryIH*, *cryIIA*, *cryIIB*, *cryIIIA*, *cryIIIB*, *cryIIIC*, *cryIVA*, *cryIVB*, *cryIVC*, *cryIVD*, *cryV* genes, mixtures thereof and sequences constructed from parts of these *cry* genes. In particular, the crystal protein encoding DNA sequences include *cryIA(b)*, *cryIA(c)*, *cryIC*, *cryIIA*, and *cryIE*. Sequences constructed from parts of any genes include hybrid crystal encoding proteins wherein domains of two or three different crystal encoding toxins are included. These hybrid genes are known in the art and may include for example domain I and domain II of one crystal encoding gene and domain III of another crystal toxin encoding gene. In particular, domain III of *cryIC* is preferred. The hybrid G27 is one such example wherein the gene includes domain I and II of *cryIE* and domain III of *cryIC*. The protein G27 is further described in Bosch et al., *Biotechnology* 12:915-918 (1994) the contents of which are hereby incorporated by reference. However, one skilled in the art can envisage various combinations of toxins comprising a hybrid toxin encoding gene and these combinations are incorporated into the invention. The terms foreign or exogenous protein or gene are terms used in the art to denote a gene which has been transferred to a host cell from a source other than the host cell.

According to this invention, the most preferred hosts include *B. thuringiensis* subspecies, and particularly subspecies *thuringiensis*, *kurstaki*, *dendrolimus*, *galleriae*, *entomocidus*, *aizawai*, *morrisoni*, *tolworthi* and *israelensis*, and most particularly *B. thuringiensis kurstaki*.

The DNA segment may further comprise an origin of replication for a gram-negative bacterium. Any origin of replication capable of functioning in one or more gram negative bacterial species or strains of *Enterobacter*, *Nitrosomonas*, *Pseudomonas*, *Serratia*, *Rhizobium*, and *Azotobacter* genera among others may be used. After cloning the DNA segment in a gram-negative bacterium such as *E. coli* and transforming a *Bacillus thuringiensis*, the only remaining exogenous insecticidal DNA sequences will be those integrated into the host's chromosome. Since the gram negative origin of replication will not function in a *Bacillus thuringiensis* host, a host transformed with the DNA segment will neither replicate nor express the crystal toxin encoding genes unless the DNA segment becomes integrated into the host chromosome.

The DNA segment may further comprise other nucleic acid sequences including selectable markers. In general, selectable markers for drug resistance, chemical resistance, amino acid auxotrophy or prototrophy or other phenotypic variations useful in the selection or detection of mutant or recombinant organisms can be used.

Preferably the DNA segment comprises an origin of replication from a gram negative bacterium and a selectable marker.

Other sequences may also be incorporated into the DNA segment including but not limited to regulatory sequences capable of directing transcription and translation of the crystal toxin encoding sequences within the host cell, such as promoters, operators, repressors, enhancer sequences, ribosome binding sites, transcription initiation and termination sites and the like. Specific examples include the *CryIC* promoter, *CryIA(c)* terminator and *ermC* promoter. Additionally, sequences adjacent to the claimed *spoVBt1* gene may be included. These sequences comprise promoter sequences, downstream enhancer sequences and the like.

The *spoV* DNA sequence suitable for use in the invention is substantially homologous to a nucleotide fragment of the *B. thuringiensis* chromosome. This fragment will generally be part of a sporulation gene and it serves as an integration site for the DNA segment of the invention into the host DNA by homologous recombination thereof with the bacterial DNA. The DNA segment of the invention may be provided as either a circular, closed DNA segment wherein homologous recombination occurs by means of a single cross over event or as a linear DNA segment wherein homologous recombination occurs by means of a double-cross over event. Thus the substantially homologous DNA sequences may be as one or two flanking DNA sequences. The *spoV* DNA sequences are homologous to a fragment of the bacterial chromosome in the range of about 15 - 1600 nucleotide bases and more preferably 200 - 1200. One skilled in the art will also recognize that at the integration site, multiple insertions can occur.

The term homologous as used herein in the context of nucleotide sequences means the degree of similarity between the sequences in different nucleotide molecules. Therefore two nucleic acid molecules which are 100% homologous have identical sequences of nucleotides. A substantially homologous nucleotide sequence or fragment is one wherein the sequences of the fragment are at least 90% and preferably 95% identical. Homologous recombination is defined as general recombination which occurs between two sequences which have fairly extensive regions of homology; the sequences may be in different molecules.

The DNA segment of the invention may be carried on a phage or a vector, a preferred vector is a plasmid and in particular the plasmids disclosed herein and in PCT International application WO 9425611, published March 19, 1995 which is hereby incorporated by reference in its entirety. Additionally, the DNA segment may be carried on a hybrid

shuttle vector for gram-positive bacteria. Appropriate vectors include any vector capable of ϕ II-replication in gram-negative bacteria, yeast's or any monocellular host in addition to gram-positive bacteria. Such shuttle vectors are known in the art.

Transformation, the process in which exogenous DNA is taken up by a recipient *B. thuringiensis* may be conducted by techniques known in the art and includes transfection, electroporation, transduction, or conjugation. Particularly preferred methods include electroporation and transduction. Host isolation may be conducted by selecting from the selectable marker on the transformed host. Transformed host may then be amplified by known techniques.

Therefore a preferred embodiment of the present invention is a method of preparing a transformed *Bacillus thuringiensis* host expressing one to three exogenous insecticidal crystal protein encoding genes comprising

a) obtaining a DNA segment comprising

1) optionally an origin of replication from a gram negative bacterium;

2) a spoV DNA sequence selected from the group consisting of

i) a spoV Bt1 gene having the nucleotide sequence show in SEQ. ID NO.1;

ii) a nucleotide sequence encoding the protein depicted in SEQ. ID NO.2;

iii) a nucleotide sequence encoding a *Bacillus thuringiensis* sporulation protein substantially similar to the protein depicted in SEQ. ID NO.2;

iv) a nucleotide sequence which hybridizes to a complementary strand of i), ii) or iii) under stringent hybridization conditions; and

v) a truncated nucleotide sequence of i), ii) or iv) above wherein the truncated sequence includes at least 300 nucleotides and

3) a DNA sequence encoding one to three insecticidal crystal proteins;

b) introducing said segment into a *Bacillus thuringiensis* host;

c) allowing homologous recombination between the DNA segment and a substantially homologous nucleotide fragment of a sporulation gene in the host *Bacillus thuringiensis* chromosome wherein the DNA segment is stably integrated into the *Bacillus thuringiensis* host chromosome; and

d) isolating stably transformed *Bacillus thuringiensis* transformants wherein said stable transformed *Bacillus thuringiensis* is capable of producing the exogenous insecticidal crystal proteins.

Also included in the invention is the transformed *Bacillus* host and progeny thereof formed by amplification of said transformant.

Mutation of a sporulation gene or genes may cause the formation of mutant spores. Mutant spores as used herein include spores from oligosporogenic and asporogenic strains. Asporogenic *B. thuringiensis* strains are those wherein spores are not formed because the strain is not capable of forming spores. Alternatively, oligosporogenic *B. thuringiensis* strains are those wherein spores are formed however, the spores may not be viable for a variety of reasons or the spores are viable but they are sensitive to heat, cold or organic solvents and rendered nonviable upon exposure thereto. Frequently, oligosporogenic *B. thuringiensis* produce what is known in the art as phase grey spores.

Mutation of a gene may occur in a number of ways well known to those in the art and include chemical mutagenesis, point mutations, deletions, insertional mutations, including use of transposons, and the like.

One embodiment of the present invention is a method of using a DNA segment of the invention in a manner to interrupt the chromosomal DNA encoding for sporulation genes. In this respect the DNA segment includes a mutated spoV DNA sequence.

A mutated spoV DNA sequence is a spoV DNA sequence of the invention wherein the sequence is altered with point mutations, deletions, or inserts. Point mutations are generally understood to mean any mutation involving a single nucleotide including the gain or loss of a nucleotide resulting in a frame shift mutation as well as transition and transversion mutations. The point mutations can occur at various codons. Preferred point mutations are used to create stop codons and may be used to destroy the ribosome binding site and methionine start codons. These stop codons can occur anywhere throughout the gene, however, they do not interrupt the process of homologous recombination between the DNA segment according to the invention and the substantially homologous chromosomal sporulation gene locus.

Accordingly, another embodiment of the present invention is an isolated mutated spoV DNA sequence selected from the group consisting of

i) the nucleotide sequence of SEQ ID No. 1;

ii) a nucleotide sequence encoding a *Bacillus thuringiensis* protein as depicted in SEQ ID No. 2;

iii) a nucleotide sequence encoding a *Bacillus thuringiensis* sporulation protein substantially similar to the protein depicted in SEQ ID No. 2;

iv) a nucleotide sequence which hybridizes to a complementary strand of a sequence of i), ii) or iii) under stringent hybridization conditions and

v) a truncated nucleotide sequence of i), ii), iii) or iv) above wherein said truncated part of the nucleotide sequence includes at least 300 nucleotides,

wherein the nucleotide sequences of i), ii), iii), iv) or v) above has one or more point mutations, inserts or deletions.

A mutated *spoV* DNA sequence may include 1 to about 25 stop codons although either a greater number than 25 or less than 25 can be used. A specific mutated *spoV* DNA sequence of the invention is part of the *spoVBt1* sequence of SEQ. ID NO.1 including nucleotide sequence 465 to 1256 inclusive wherein the following nucleotides as illustrated in Table 1 have been altered. In general, stop codons should be engineered before nucleotide 1404 of SEQ ID No.1, or a related *spoV* DNA sequence to prevent reversion to wild type spores in the recipient host cells. Additionally, the peptide encoded by a mutated *spoV* DNA sequence should be less than 306 amino acids. Most preferably stop codons should be engineered before nucleotide 1256 of SEQ ID No.1 or a related *spoV* DNA sequence.

TABLE 1

Nucleotide #	original codon	altered to
465	G	T
475	T	A
487	T	A
492	A	T
873	G	T
881	C	A
1243	T	A
1254	A	T

This specific mutated *spoV* DNA sequence is designed (m) *spoVBt1-8*.

A mutated *spoV* DNA sequence may include the point mutations described above or a sequence substantially homologous to the non-mutated codons of sequence 465 to 1254.

The mutated *spoV* DNA sequence also includes a *spoV* DNA sequence which has exogenous inserts of nucleic acid sequences for example, the inserts may comprise 2 to 15 nucleotides; however, more nucleotides could be used.

Preferred mutated *spoV* DNA sequences are those wherein the sequence is a mutated sequence of SEQ ID No. 1 and said mutation consists of between one and twenty-five point mutations before nucleotide number 1404 or those wherein the sequence is a truncated mutated nucleotide sequence comprising base pair 465 through 1265 of SEQ ID No. 1 and said point mutations are between one and twenty-five.

A preferred DNA segment comprises the mutated *spoV* DNA defined above; and one to three insecticidal crystal protein encoding genes.

A further preferred DNA segment comprises the mutated *spoV* DNA sequence defined above, an insecticidal crystal protein encoding gene; and an origin of replication from a gram negative bacterium.

Therefore another preferred embodiment of the present invention is a method of preparing insecticidal crystal protein producing *Bacillus thuringiensis* strains with mutant spores comprising

a) obtaining a DNA segment comprising

1) an origin of replication from a gram negative bacterium;

2) a mutated *spoV* DNA sequence selected from the group consisting of

i) a *spoVBt1* gene having the nucleotide sequence shown in SEQ. ID NO.1;

ii) a nucleotide sequence encoding the protein depicted in SEQ. ID NO.2;

iii) a nucleotide sequence encoding a sporulation protein substantially similar to the protein depicted in SEQ. ID NO.2;

iv) a nucleotide sequence which hybridizes to a complementary strand of i), ii) and iii) under stringent hybridization conditions; and

v) a truncated nucleotide sequence of i), ii), iii) or iv) above wherein said truncated sequence includes at least 300 nucleotides;

vi) wherein the nucleotide sequence of i), ii), iii), iv) or v) above has one or more point mutations, inserts or deletions; and

3) a DNA sequence encoding at least one insecticidal crystal toxin protein;

b) introducing said segment into a sporulating *Bacillus thuringiensis* host;

c) allowing homologous recombination between the DNA segment and a substantially homologous sporulation gene fragment of the host *Bacillus thuringiensis* chromosome wherein the DNA segment including the mutated *spoV* DNA sequence is stably integrated into the *Bacillus thuringiensis* host chromosome; and

d) isolating stably transformed *Bacillus thuringiensis* transformants wherein said stable transformed *Bacillus thuringiensis* produces mutant spores and is capable of producing the exogenous crystal toxin protein(s).

The substantially homologous sporulation gene fragment in the host chromosome is preferably the *spoVBt1* chromosomal fragment.

Also provided are transformed *Bacillus thuringiensis* hosts produced as described above.

The method further comprises employing the transformed aliosporogenic or asporogenic host.

Transduction is a virus mediated transfer of host DNA from one host cell (a donor) to another cell (recipient). When a phage replicates in a donor cell, a few progeny virions encapsidate pieces of the host DNA in addition to phage DNA. These virions can adsorb to a new host cell and introduce their DNA in the usual way. In this invention, the host strain which is transformed with a DNA segment of the invention can be further transduced with a phage. The host (donor) DNA which is incorporated into the phage undergo recombination with a homologous region of a recipient's chromosome so that the genes can be stably inherited. This is generally referred to by those skilled in the art as generalized transduction. Phages are known by those skilled in the art and include all phages capable of infecting *B. thuringiensis* strains, for example CP-51 and CP-51ts45 and all derivations thereof. In the present invention, the preferred recipient cell is from a strain of *Bt kurstaki*.

Therefore, in another aspect the invention is a method of transducing a transformed *Bacillus thuringiensis*, prepared by either one of the methods described above, comprising

a) exposing a *Bacillus thuringiensis* host of the invention to a transducing phage;

b) allowing the phage to replicate in the host wherein one to three exogenous crystal protein encoding genes integrated into the host chromosome are incorporated into the phage or allowing the phage to replicate in the host *Bacillus thuringiensis* wherein the mutated *spoV* DNA sequence and one to three exogenous crystal protein encoding DNA proteins integrated into the host chromosome are incorporated into the phage; and

c) introducing the exogenous crystal protein encoding sequences from the phage into a recipient *Bacillus thuringiensis* strain

wherein said introduced exogenous crystal protein encoding DNA sequences are stably incorporated into the recipient *Bacillus thuringiensis* chromosome and expressed in said recipient or wherein the mutated *spoV* DNA sequence and said exogenous crystal protein encoding DNA sequences are stably incorporated into the recipient *Bacillus thuringiensis* chromosome and the exogenous crystal protein is expressed in said recipient wherein the recipient produces mutant spores.

The mutated *spoV* DNA sequence and exogenous crystal protein encoding DNA sequence are preferably incorporated at a sporulation gene chromosomal fragment.

The DNA segment to be integrated in the host chromosome comprises preferably a truncated mutated *spoV* DNA sequence including nucleotide sequence 465 to 1265 of SEQ ID No. 1 with one to twenty-five point mutations and the crystal toxin encoding DNA *cryIA(b)*, *cryIA(c)*, *cryIC*, *cryIIA* or *cryIE* and sequences constructed from parts thereof.

The invention also provides a transduced strain obtained as described above.

5 The recipient *Bacillus thuringiensis* may be rendered oligosporogenic or asporogenic depending on the *spoV* DNA sequence used in the DNA segment introduced into the transformed host. A most preferred locus for chromosomal integration is the *spoVBT1* nucleotide fragment of the recipient *Bacillus thuringiensis* strain. However, other sporulation gene fragment may equally serve as a chromosomal locus. Preferred sporulation gene fragment include those substantially homologous with a *spoV* DNA sequence.

10 The stable incorporation of the DNA segment according to the invention into a host chromosome is defined as the maintenance of the DNA segment within the host chromosome through many generations.

The invention further relates to pesticidal compositions, preferably insecticidal compositions, wherein the transformed *Bacillus thuringiensis* or protein derived from said *Bacillus* are the active ingredient. The compositions of the invention include an asporogenic or oligosporogenic *Bacillus thuringiensis* encoding one or more insecticidal Cry proteins and is applied at an insecticidally effective amount. An insecticidally effective amount is defined as will vary depending on such factors as the specific Cry protein, specific insects to be controlled, the specific plant to be treated, and the method of applying the insecticidally active compositions.

15 The compositions of the invention may contain about 10^6 to about 10^{13} microorganisms per gram ca. The pesticidal concentration will vary depending on the carrier of the particular formulation. The compositions contain from 0.1 to 99% of the transformed host or progeny thereof and 0 to 99.9% of a solid or liquid carrier.

20 The insecticidal compositions of the invention may be formulated with an agriculturally acceptable carrier. The formulated compositions may be in the form of dusts, granular material, suspensions in oil or water, emulsions or as wettable powders. Suitable agricultural carriers may be solid or liquids and are well known to those in the art. Agriculturally acceptable carriers as used herein include all adjuvants such as wetting agents, spreaders, emulsifiers, dispersing agents, foaming agents foam, suppressants, penetrants, surfactants, solvents, solubilizers, buffering agents, stickers etc., that are ordinarily used in insecticide formulation technology. These are well known to those skilled in the art of insecticide formulation.

The formulations comprising the asporogenic or oligosporogenic *Bacillus thuringiensis* strains and one or more liquid or solid adjuvants are prepared in a manner known to those in the art.

30 The compositions of this invention are applied to the locus where control is desired and typically onto the foliage of a plant to be protected by conventional methods. These application procedures are well known in the art. The formulations of the present composition may be applied by spreading about 10^8 to about 10^{16} spores per acre. With oligosporogenic or asporogenic compositions the spores may be present but are either immature or non-viable. The compositions are best applied as sprays to plants with subsequent reapplication. Plants to be protected within the scope of the invention include but are not limited to cereals, fruits, leguminous plants, oil plants, vegetable plants, deciduous and conifer trees, beet plants, ornamentals. The compositions may be effective against pests of the orders Coleoptera, Lepidoptera and Diptera.

35 Yet another embodiment of the present invention is a method of using a *Bacillus thuringiensis* chromosomal sporulation gene fragment as a locus for chromosomal integration of a DNA segment, said segment comprising one to three insecticidal crystal protein encoding genes wherein said gene is stably integrated into the *Bacillus thuringiensis* chromosome.

The chromosomal gene fragment is preferably substantially homologous to about 15 to about 1600 nucleotides on the DNA segment and the DNA segment includes DNA sequences encoding a *Bacillus thuringiensis* sporulation protein.

45 The stable integration preferably renders the *Bacillus thuringiensis* and any progeny thereof an oligosporogenic or asporogenic strain.

The *Bacillus* is preferably a strain of *Bacillus thuringiensis* *kurstaki*.

The methods of the present invention make use of techniques of genetic engineering and molecular cloning that are known to those skilled in the art using commercially available equipment and are included in Maniatis, et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1991).

50 The present invention will now be described in more detail with reference to the following specific, non-limiting examples.

EXAMPLES**EXAMPLE 1: Identification and Cloning of *B. thuringiensis* *spoV Bt1* gene:**5 **A. Preparation of transposon Tn917 bearing plasmid pLTV1.**

Plasmid pLTV1 is isolated from the *B. subtilis* strain PY1177. The strain is grown overnight (18-20 hours) on TBAB plates (3.3% Difco Tryptose Blood Agar Base) containing 0.5% glucose and Tet¹⁰ (10 µg/ml). Cells from single colonies are used to inoculate 10 ml LB (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, 0.5% NaCl, pH 7.0) containing 0.5% glucose and Tet¹⁰ (10 µg/ml). The cells are incubated for 5 hours with shaking (300 rpm) at 37°C, centrifuged at 18,500 x g and 4°C for 10 minutes, then washed once in 10 ml of SET buffer [20% sucrose, 50 mM disodium ethylenediaminetetra-acetic acid (EDTA), 50 mM Tris-HCl pH 8.0]. The pellet is resuspended in 500 µl SET solution containing 2 mg/ml of lysozyme and 0.4 mg/ml RNase A (Boehringer Mannheim Biochemicals, Indianapolis, IN). The cell suspension is incubated at 37°C for 10 minutes and 1 ml of the lysis mixture [1% sodium dodecyl sulfate (SDS), 200 mM NaOH] is added, followed by 725 µl of prechilled neutralization buffer (1.5 M potassium acetate pH 4.8). The mixture is then incubated on ice for 20 minutes; centrifuged at 18,500 x g and 4°C for 10 minutes; and the supernatant is transferred to a fresh tube. Plasmid DNA is then isolated using a Mini Qiagen Plasmid Kit (Qiagen Inc., Chatsworth, CA).

20 **B. Transfer of pLTV1 to *E. coli* GM2163.**

Unless otherwise indicated, *E. coli* and *B. thuringiensis* strains are grown at 37°C and 30°C, respectively. Plasmid pLTV1 requires conditioning in a dcm(-) host cell prior to transformation of *B. thuringiensis*. This is accomplished by transfer of pLTV1 into dcm(-) *E. coli* GM2163 (New England Biolabs, Inc., Beverly, MA). Competent *E. coli* GM2163 cells are prepared by inoculating a single colony into 30 ml SOB medium (2% Bacto Tryptone, 0.5% Bacto Yeast Extract, 0.06% NaCl, 0.05% KCl, 10 mM MgCl₂, 10 mM MgSO₄). The cells are incubated overnight at 300 rpm and 37°C. Two hundred ml of SOB media, in a 2 L flask, is inoculated with 8 ml of the overnight culture, and incubated at 37°C and 300 rpm to an OD₅₅₀ of 0.3. The culture is placed on ice for 15 min., centrifuged at 4,000 x g and 4°C for 5 minutes, and the pellet gently resuspended in 64 ml of transformation buffer 1 (1.2% RbCl, 0.99% MnCl₂·4H₂O, 30 mM potassium acetate pH 5.8, 0.25% CaCl₂·2H₂O, 15% glycerol). After a 15 minute incubation on ice, the cells are again centrifuged at 4,000 x g and finally resuspended in 16 ml of transformation buffer 2 (10 mM MOPS pH 7.0, 0.12% RbCl, 1.1% CaCl₂·2H₂O, 15% glycerol). Approximately 50 µl of competent cells and 4 µl DNA are mixed in a 1.5 ml Eppendorf tube and incubated on ice for 1 minute. The mixture of cells and DNA (pLTV1 isolated from *B. subtilis* strain PY1177) are transferred to a prechilled 0.2 cm gap electrode cuvette and pulsed using the high voltage Gene Pulser electroporation apparatus. The electroporation conditions were 25 µF, 2.5 kV, and 200 Ω. Cells are immediately transferred to 1 ml SOC medium (2% Bacto Tryptone, 0.5% Bacto Yeast Extract, 0.06% NaCl, 0.05% KCl, 20 mM glucose) and incubated at 37°C and 225 rpm for 1 hr. The cells are plated on LB agar containing Amp⁷⁵ (75 µg/ml) and incubated overnight at 37°C. Plasmid pLTV1 is isolated from transformed *E. coli* GM2163 cells using the Mini Qiagen Plasmid Kit.

40 **C. Transfer of pLTV1 to *B. thuringiensis* Cry-B.**

The *B. thuringiensis* strain HD1Mit9 was used for transposon mutagenesis. This strain was obtained from Dr. Arthur I. Aronson at Purdue University. It is an acrySTALLIFEROUS derivative of *B. thuringiensis* subspecies *kurstaki* HD1 and contains only one 4-mDa plasmid.

Plasmid pLTV1 isolated from *E. coli* is unstable in HD1Mit9. As a result, the plasmid DNA from GM2163 is transformed into Cry B, a plasmid cured crystal-minus strain of *B. thuringiensis* (Stahly, D.P., Dingmann, D.W., Bulla, L.A. and Aronson, A.I., Biochem. Biophys. Res. Com. 84:581-588, 1978). To prepare competent cells, CryB is grown overnight on an LB plate. Individual colonies are used to inoculate 100 ml of BHIS medium (3.7% Brain Heart Infusion, 0.5 M sucrose) in a 1 L flask. The culture is incubated at 37°C with shaking, until an OD₆₀₀ of 0.2-0.3. The cells are transferred to a prechilled 250 ml bottle and centrifuged for 7 minutes, at 6,500 x g and 4°C. The pellet is washed once in 100 ml and twice in 10 ml of ice cold HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])/sucrose wash solution (5 mM HEPES, pH 7.0, 0.5 M sucrose). Cells are then resuspended in a solution containing 10 ml of HEPES/sucrose solution and 2.5 ml of 50% glycerol. Competent *B. thuringiensis* cells (200 µl) are mixed with 10 µl of plasmid DNA pLTV1 (1-5 µg) in a prechilled 0.2 cm gap electrode Gene Pulser Cuvette, and exposed to an electrical current in the Gene Pulser electroporation apparatus (Bio-Rad Laboratories, Richmond, CA). The parameters for the electroporation of CryB are 1.05 kV, 25 µF, Ω = ∞. Following electroporation the cells are immediately transferred to 5 ml BHIS in a 125 ml flask and grown at 30°C and 250 rpm. After three hours of growth, the cells are transferred to LB agar plates containing Tet¹⁰. The plates are incubated overnight at 30°C and the transformants, designated CryB(pLTV1) are restreaked onto fresh LB Tet¹⁰ plates.

D. Isolation of Plasmid pLTV1 from *B. thuringiensis* Strain Cry-B.

Cry-B(pLTV1) is streaked onto LB Tet¹⁰ plates. The culture is grown overnight. A single colony is restreaked onto an SA plate (1x Spizizens salts, 1% casamino acids, 0.5% glucose, 0.005 mM MnSO₄·H₂O, 1.5% Bacto agar) and incubated for 3-4 hours at 37°C. The 1x Spizizens salts contain 0.2% (NH₄)₂SO₄, 1.4% K₂HPO₄, 0.6% KH₂PO₄, 0.1% Sodium-Citrate 2H₂O, and 0.02% MgSO₄·7H₂O (Anagnostopoulos and Spizizen, 1961). The grown cells are removed from the plate, resuspended in 100 µl TESL (100 mM Tris-HCl pH 8.0, 10 mM EDTA, 20% sucrose, 2 mg/ml lysozyme) and incubated at 37°C for 30-60 minutes. Two hundred microliters of lysis solution (200 mM NaOH, 1% SDS) is added to the tube followed by a 5 minute incubation at room temperature. After addition of 150 µl ice-cold 3 M potassium acetate pH 4.8, the suspension is microcentrifuged for 20 minutes at 18,500 x g and 4°C. The supernatant is transferred to a fresh tube and mixed with 1 ml of 100% ethanol. This suspension is left at -20°C for 1 hour and centrifuged at 18,500 x g and 4°C for 30 minutes. The plasmid DNA is washed with 70% ethanol, dried under vacuum, and resuspended in 20 µl of TE.

E. Transfer of pLTV1 to *B. thuringiensis* Strain HD1 Mit9.

Plasmid pLTV1 isolated from Cry-B(pLTV1) is introduced into strain HD1Mit9. *B. thuringiensis* HD1Mit9 cells (Dr. Arthur I. Aronson, Purdue University) are made competent and transformed using the same procedure described above for Cry-B. The plasmid is isolated from HD1Mit9(pLTV1) using the same protocol used for Cry-B(pLTV1). However, the parameters for the electroporation of HD1Mit9 are 1.2 kV, 3 µF, $\Omega = \infty$. The presence of plasmid pLTV1 in the HD1Mit9 transformants was confirmed by restriction enzyme digestion and polymerase chain reaction (PCR). The results of each experiment are analyzed by agarose gel electrophoresis. The pLTV1 DNA (1 µg), isolated from HD1Mit9, is digested with *EcoRI* (Pharmacia Biotechnology, Piscataway, NJ) under the conditions described by the manufacturer. Primers LacNHS1, SEQ ID No. 3 and LacNHS2, SEQ ID NO. 4, used for the PCR reactions, are synthesized on a PCR-Mate DNA synthesizer model 391 (Applied Biosystems, Foster City, CA).

Primer	Sequence (5'-3')	SEQ ID
<u>NO.</u>		
LacNHS1	GGCTTTTCGCTACCTGGAGAGACGCGCCCCGC	3
LacNHS2	CCAGACCAACTGGTAATGGTAGCGACCGGC	4

A single *B. thuringiensis* colony is resuspended in 15 µl sterile water, placed in a boiling water bath for 10 minutes, and then centrifuged for 5 minutes. Each PCR reaction contained 2 µl of the supernatant from the boiled cells, 1X PCR buffer [100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.1% (wt/vol) gelatin], 200 µM deoxyribonucleoside triphosphates (dNTP's; 1.25 mM of dATP, dCTP, dGTP, and dTTP), 1 µM LacNHS1 primer, 1 µM LacNHS2 primer, and 2.5 units AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The reactions are run in a DNA Thermal Cycler (Perkin-Elmer Cetus) for 25 cycles. Each cycle consists of 1 minute at 94°C (denaturation), 2 minutes at 55°C (hybridization), and 3 minutes at 72°C (extension). The PCR products are analyzed by agarose gel electrophoresis. Synthesis of a 1.5 kb PCR generated fragment indicates that the clone carried the pLTV1 plasmid.

F. Preparation of Tn917 Insertion Libraries.

To prepare the library, HD1 Mit9 (pLTV1) is grown on an LB plate containing Tet¹⁰. Two to three colonies are used to inoculate 10 ml Penassay broth (1.75% Difco Antibiotic Medium 3) containing Ery^{0.15}. After 90 minutes of growth at 30°C and 300 rpm, the concentrations of antibiotics in the culture are increased to Ery¹ and Lm²⁵. When the culture reaches an OD₅₉₅ of 0.5, a 100 µl portion is added to 10 ml fresh Penassay broth containing Ery¹ and Lm²⁵. After 16 hours of growth at 39°C and 300 rpm, the culture is diluted 1:15 with 10 ml Penassay broth containing Ery¹ and Lm²⁵ and grown with moderate shaking at 39°C until an OD₅₉₅ of 2.0. The cells are pelleted by centrifugation (4,000 x g, 4°C), resuspended in 500 µl of Penassay broth containing Ery¹ and 30% glycerol, and frozen on dry ice. Dilutions (10⁻³ and 10⁻⁴) of the library are plated onto Penassay Ery⁵ plates and incubated at 39°C for 16 hours. Individual colonies are patched onto LB plates each containing different antibiotics (Tet¹⁰, Lm²⁵, and Ery⁵) and grown overnight at 30°C. Those colonies that grow only on LB Lm²⁵ and LB Ery⁵ but not LB Tet¹⁰ contained Tn917 insertions. These colonies are grown on CYS plates (1% Casitone, 0.5% glucose, 0.2% Bacto Yeast Extract, 0.1% KH₂PO₄, 0.5 mM MgCl₂, 0.05 mM MnCl₂, 0.05 mM ZnSO₄, 0.05 mM FeCl₂, 0.2 mM CaCl₂, 1.5% Bacto agar) and examined microscopically for their ability to sporulate. Over 1 x 10⁴ colonies are screened for transposon insertions. Sixty-three colonies containing a

chromosomal insertion are obtained. Only three colonies (5% of the insertion mutants) are sporulation-defective. The sporulation mutants are further analyzed.

To identify auxotrophs and citric acid cycle-defective colonies, the *B. thuringiensis* sporulation mutants are grown on glucose minimal and lactate minimal plates for 1-2 days, and the growth is compared to that of HD1Mit9. Glucose minimal agar is made of 1x salt solution [1 liter, 5.6 g K_2HPO_4 , 2.4 g KH_2PO_4 , 1.2 g $(NH_4)_2SO_4$, 0.4 g sodium citrate, pH 7.0], 0.005 mM $FeCl_2$, 0.25 mM $MgCl_2$, 0.96% glucose, 0.0002 mM $MnCl_2$, 0.00012% Thiamine-B1, and 1.5% Bacto agar. Lactate minimal medium contains 0.2% lactate and 25 mM glutamate instead of glucose and sodium citrate. The three sporulation mutants grow well on glucose and lactate minimal media indicating that they are not auxotrophs or defective in citric acid cycle enzymes.

G. Isolation of DNA from the *B. thuringiensis* Sporulation Mutant.

The sporulation mutant strain, HD1Mit9::Tn917, is grown in 2 ml LB overnight at 200 rpm. The overnight culture is used to inoculate 100 ml LB (1% inoculation). The cells are grown at 300 rpm to an OD_{600} of 0.7-1.0, collected by centrifugation (3,840 x g, 5 minutes, 4°C), and resuspended in 5 ml TES (25 mM Tris-HCl pH 8.0, 25 mM EDTA, 25% sucrose). The cell suspension is mixed with 0.55 ml of 10 mg/ml lysozyme in TES solution and incubated at 37°C for 60 minutes. SDS is added to 2% weight-to-volume, followed by a 15 minute incubation at 50°C. The suspension is mixed with 1.52 ml of 5 M NaCl and incubated at 50°C for 5 minutes. The sample is incubated at 0°C for 1 hour, centrifuged for 10 minutes at 18,500 x g and 4°C, and the supernatant is transferred to a new tube. To purify the DNA from the protein, the supernatant is treated with phenol and chloroform. First, it is mixed with 5 ml TE-equilibrated phenol followed by 15 minutes incubation at 50°C, then with 5 ml phenol/chloroform (1:1), and finally with 5 ml chloroform. The aqueous phase is separated from the organic phase by centrifugation (4,000 x g, 5 minutes) at each step. The DNA is precipitated by the addition of 4.6 ml isopropanol and centrifugation (18,500 x g, 30 minutes, 4°C). The air-dried DNA pellet was dissolved in 2 ml of TE and stored at -20°C.

H. Cloning the Chromosomal DNA Adjacent to Transposon Insertions.

B. thuringiensis chromosomal DNA is cloned and maintained in *E. coli* DH5 α (Gibco BRL, Grand Island, NY) or HB101 (New England Biolabs, Inc.) strains. Restriction enzymes, T4 DNA Ligase, and reaction buffers are purchased from New England Biolabs, Inc., Pharmacia Biotechnology (Piscataway, NJ), or Gibco BRL. Competent *E. coli* cells were prepared as follows. The strains are incubated on LB agar plates for 16-18 hours. Several colonies are used to inoculate 100 ml LB in 1 L Erlenmeyer flasks. Liquid cultures are grown at 37°C and 300 rpm. When the cultures reach an OD_{600} of 0.2, they are placed on ice for 15 minutes and then centrifuged for 10 minutes at 5,500 x g and 4°C. The cells are resuspended in 50 ml (1/2 volume) 0.05 M $CaCl_2$, incubated on ice for 20 minutes, and centrifuged as described above. The competent cells are resuspended in 5 ml (1/20 volume) 50 mM $CaCl_2$ containing 20% glycerol and placed into microfuge tubes (100 μ l per tube).

B. thuringiensis chromosomal DNA (11-14 μ g) is digested with a restriction enzyme as follows. Reactions contain 30-50 units of enzyme in a 1x reaction buffer and are incubated overnight at 37°C. The 10x reaction buffers include REact 2 (500 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$, 500 mM NaCl), REact 3 (500 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$, 1 M NaCl), and NEB 3 (500 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 M NaCl). The REact 2, REact 3, and NEB 3 buffers are used for the *Xba*I, *Eco*RI, and *Bsp*EI enzymes, respectively. The enzymes are inactivated by heating at 70°C for 40 minutes. Digested DNA is ligated in a 100 μ l volume using 16 units of T4 DNA Ligase and 20 μ l of 5x Ligase Reaction Buffer [50 mM $MgCl_2$, 25% (wt/vol) polyethylene glycol 8000, 5 mM ATP, 5 mM DTT, 250 mM Tris-HCl pH 7.6] and incubated overnight at 16°C. The ligated mixtures are used to transform 100 μ l *E. coli* HB101 competent cells. Ampicillin-resistant transformed colonies are isolated after 16 hours of growth on LB agar containing Amp^r. Plasmid DNA is extracted from the HB101 transformants, transferred to *E. coli* DH5 α or GM2163, and then analyzed by restriction endonuclease digestion.

I. Determination of the Nucleotide Sequence of *spoVBt1*.

Both strands of the cloned gene are sequenced using the primers listed in Table 1 which are synthesized on a PCR-Mate DNA synthesizer model 391. The positions of the oligonucleotides are shown in Figure 2. A Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH) is used to sequence the cloned *B. thuringiensis* chromosomal fragments as described by the manufacturer. The sequence of *spoVBt1* gene is shown in SEQ ID NO.1. The molecular mass of the *spoVBt1* gene product is 36.7 kDa.

Table 2:Primers used for sequencing *B. thuringiensis* *spoVBt1* gene

	Primer	Sequence (5'-3')	SEQ ID NO.
5			
10	MS1	GAGAGATGTCACCGTCAAG	5
	MS2	CCCTGTACCTGGATTCCC	6
	MS3	GGGAATCCAGGTACAGGG	7
15	MS4	CCATCCCAACAAGCTTCCC	8
	MS5	GGGAAGCTTGTTGGGATGG	9
20	MS6	CCTGTCCCCCTTGTAATGC	10
	MS7	GCATTACAAGGGGACAGG	11
	MS8	CGCCGTCTACTTACAAGCAGC	12
25	MS9	GGTGGTGGGACTATGGAG	13
	MS10	CTCCATAGTCCCACCACC	14
30	MS11A	CGAGGAGGAGAGAAGGAC	15
	MS11B	GTCCTTCTCTCCTCCTC	16
	MS12A	CGAAGTGACGGTCTGG	17
35	MS12B	CACGATGCATCG	18
	MS13A	CGAAAGAGGCTGAATGG	19
40	MS13B	GGGCGGTATGTACGG	20
	MS13C	CCGTACATACGCCC	21
	MS14	GCATCAAATCCATACTCGATATTCC	22
45	MS14A	CGAGTATGGATTTGATGCTCG	23
	MS16	GGACACGATCCTAATTCAGC	24
50	MS16A	GCTGAATTAGGATCGTGTCC	25

J. Characterization of Spores from the sporulation mutant strain HD1Mit9::Tn917.

The resistance properties of the mutant and the wild type spores of HD1Mit9 against heat, lysozyme, and organic solvents are compared. The wild type and mutant strains are grown in 100 ml CYS medium. The cultures are harvested 48 hours after they reached stationary phase. The sporulated cultures are then exposed to various treatments, and serial dilutions in 0.1% peptone are plated onto LB agar and incubated overnight at 30°C. Colonies arising from germinated spores are counted. Resistance of spores to heat, lysozyme, and organic solvents is determined. Heat Treat-

ment: Cultures are diluted 1:10 in 0.1% sterile peptone and divided into two equal parts. One part is incubated at 55°C and the other at 65°C for 45 minutes with occasional mixing. Lysozyme Treatment: Cultures are diluted 1:100 in 0.1% peptone containing 250 µg/ml lysozyme and incubated at 37°C for 15 minutes. Organic Solvent Treatment: Samples are treated with toluene, 1-octanol, and chloroform using the following protocol. One milliliter of the cultures is mixed with 7 ml of 0.1% peptone and 2 ml of organic solvent. The mixtures are then vortexed for 1 minute. For the acetone treatment, the cultures are diluted 1:10 in acetone and incubated at room temperature for 15 minutes. The mutants spores are sensitive to heat and organic solvents and resistant to lysozyme. The relative resistance of spores produced by the sporulation mutant strain, from the most resistant to the least resistant, are as follows: lysozyme, heat, toluene, chloroform, acetone, and 1-octanol.

EXAMPLE 2: Chromosomal Integration

A. Preparation of the *spoVBt1* DNA sequence and a mutated *spoV* DNA sequence.

The *spoVBt1* DNA sequence is prepared as described above in Example 1. Primers MS18, MS19A, MS19B and MS20 are used to PCR amplify a mutated *spoV* gene using the native *spoVBt1* as a template. The primers are designed to contain several point mutations such that the putative ribosomal binding site and the start codon are destroyed and multiple stop codons are introduced throughout the *spoVBt1* sequence. This mutated *spoV* DNA sequence corresponds to nucleotide sequence 465 to 1256 of SEQ ID No.1 with alterations as indicated in Table 1. The specific mutated *spoV* DNA sequence is designated (m) *spovBt1-8*.

Primer	Sequence (5'-3')	SEQ ID
<u>NO.</u>		
MS18	GATGTGATTGTAAGGAACAATCGAAGCGATAGAAAAAC	26
MS19A	GATCTTGTATGAGAGTAAATCGGCCATACAGC	27
MS19B	GCTGTATGGCCGATTACTCTCATACAAGCTC	28
MS20	CTATACAGCATGTTAATGATCCC	29

To obtain the (m)*spovBt1-8* gene, two PCR reactions are involved. The two fragments of the gene, the 3' and the 5' halves of the sequence, containing a 32 bp overlap corresponding to MS19A and MS19B on each DNA strand are amplified. The 5' half is amplified using primers MS18 and MS19B and the 3' half is amplified using primers MS19A and MS20. The two fragments are mixed, denatured and annealed. The entire (m)*spovBt1-8* gene is amplified using primers MS18 and MS20.

B. Chromosomal Integration of crystal genes at the *spoV* gene *Bt1* site with the (t)*spovBt1-1*.

Crystal genes are integrated into the *B. thuringiensis* chromosome using the pSB1209 plasmid (Figure 6). The plasmid pSB901 (Figure 3) is constructed to provide an erythromycin resistance gene, *ermC*. The *ermC* gene is isolated as a *HindIII/ClaI* fragment from the p1M13 *Bacillus subtilis* plasmid described by Monod et al. [Monod et al., J. Bacteriol. 167:138-147 (1986)]. The *ermC HindIII/ClaI* fragment is ligated to pUC18 cut with *HindIII* and *AclI*. To replace the tetracycline resistance gene (*tet^r*) in pBR322 (Figure 3) with the *ermC* gene from pSB901, pBR322 is digested with *AvaI* and the linearized vector is treated with the Klenow fragment of *E. coli* DNA polymerase I to generate a blunt end. Following Klenow treatment, pBR322 is digested with *HindIII* and the large fragment is purified away from the *tet^r* gene fragment. Plasmid pSB901 is digested with *SmaI* followed by *HindIII* and the fragment carrying the *ermC SmaI-HindIII* fragment is purified. The *ermC* gene is ligated into the pBR322 *HindIII* large fragment to generate pSB140 (Figure 3).

Primer	Sequence (5'-3')	SEQ ID NO.
5 KK14	AGCTTGCGGCCGCGTCGACCCCGGGCCATGGGGGCCCCG	30
KK14B	AATTCGGGCCCCCATGGCCCGGGTTCGACGCGGCGCA	31
10 MS17	GCGAAAGAAAAACAACAATC	32

Using PCR primers, MS14, SEQ ID NO.22, and MS17, SEQ ID NO.32, (t)*spoVBt1-1* gene is amplified from *B. thuringiensis* HD73 strain. The 916 bp PCR product is blunted at both ends using the DNA polymerase I, Klenow fragment, and cloned into plasmid pUC18 at the *SmaI* site. The (t)*spoVBt1-1* corresponds to base pair 488 through 1404 of SEQ ID NO.1. The resulting plasmid is called pSB1207 (Figure 5).

The (t)*spoVBt1-1* gene is isolated from pSB1207 using *EcoRI* and *HindIII* restriction enzymes and the ends were blunted with the Klenow fragment. The pSB210 is linearized using *HindIII* enzyme, blunted with Klenow, and dephosphorylated using Calf Intestinal alkaline phosphatase (CIP). The isolated (t)*spoVBt1-1* gene is then ligated into the linearized pSB210 plasmid. The resulting plasmid is called pSB1209 (Figure 6). Various crystal genes are cloned at the *Apal* and *NotI* sites of pSB1209 and integrated into the *B. thuringiensis* chromosome at the *spoV Bt1* site.

25 C. Chromosomal Integration of crystal genes at the *spoV Bt1* site using the (m) *spoVBt1-8* fragment.

The (m)*spoVBt1-8* fragment is amplified from *B. thuringiensis* HD73 strain using the PCR technique. The 0.8 kb PCR product is blunted at both ends using the Klenow fragment, and cloned into plasmid pUC19 at the *SmaI* site. The resulting plasmid is called pSB1218 (Figure 7).

30 The (m) *spoVBt1-8* fragment is isolated from pSB1218 at the *KpnI* and *BamHI* sites. This fragment is blunted at both ends using T4 DNA polymerase, and cloned into plasmid pSB210 at the *MscI* site. The resulting plasmids are pSB1219 (Figure 8) and pSB1220 (Figure 9). The cloned (m) *spoV Bt1-8* fragment is either in the same orientation as *ermC* gene in pSB210 (pSB1219) or in the opposite direction of the *ermC* open reading frame (pSB1220). The G27 gene encoding a *CryI/C*/*CryIE* hybrid crystal protein is cloned in pSB1219 using the following steps:

35 To construct the pSB210.1 plasmid, (Figure 11), the phospholipase C "plc" gene is added to pSB210. The DNA sequence of the plc region from *B. thuringiensis* strain ATCC 10792 is obtained from Genbank (Accession number X14178) and is described by Lechner et al., [Lechner, M., et al., Mol. Microbiol. 3:621-626 (1989)]. The plc region is amplified from HD73 total DNA by PCR using primers Phos1 and Phos4.

Primer	Sequence (5'-3')	SEQ ID NO.
40 Phos1	GGAACGCTACATACTAGTGATAGAGTAG	33
45 Phos4	GCTTGTACACCGCAACTGTTTTTCGCATG	34

50 The PCR product is cloned into the *SmaI* site of pUC18 to construct pSB139 (Figure 10). The plc target region is isolated on a 2.2 kb blunted-*KpnI*, *BamHI* fragment from pSB139, gel-purified and ligated into pSB210, which has been digested with *MscI* and *BamHI* and purified using the GeneClean Kit (Bio101), following the manufacturer's directions. The resulting plasmid is designated pSB210.1 (Figure 11).

55 The plasmid pSB32, (Figure 12) carrying the holotype *cryIA(b)* gene from *B. thuringiensis aizawai*, is cut with *Apal* and *NotI* to release the 4.2 kb fragment containing the *cryIA(b)* gene. This plasmid also contains pBlueScript KS⁺, *cryI*C promoter and *cryIA(c)* terminator which control the expression of *cryIA(b)* gene. This isolated *cryIA(b)* fragment is ligated into pSB210.1 cut with *Apal* and *NotI* to generate pSB219 (Figure 13) containing the *cryIA(b)*, the plc, and the *ermC* genes.

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A 3.9 kb *Apal*/*NotI* fragment containing a G27 toxin coding region is ligated to the 6.3 kb *Apal*/*NotI* fragment from pSB219. The resulting plasmid is called pSB458 (Figure 14).

The G27 is isolated from pSB458 using *Apal* and *NotI* digests and ligated to pSB1219 at the *Apal*/*NotI* sites. The resulting plasmid is called pSB1221 (Figure 15). This plasmid is used for integrating G27 by the transformation process described below into *B. thuringiensis* chromosome at the homologous *spoV Bt1* region while creating a mutation at the site.

Other crystal genes are also cloned at the *Apal* and *NotI* sites of pSB1219 and pSB1220 and then integrated into the *B. thuringiensis* chromosome at the homologous *spoV Bt1* region while creating a mutation at that site.

D. *B. thuringiensis* Transformation.

To prepare competent HD73 and W4D23 *B. thuringiensis* cells, strains are grown in 50 ml BHIS medium (50% brain heart infusion broth, 50% 1 M sucrose) at 37°C and 300 rpm until they reach OD₆₀₀ of 0.2-0.3. W4D23 is a crystal-minus derivative of HD73. The cells are washed successively in one volume, 1/2 volume, and 1/4th volume of ice-cold HEPES/sucrose solution. The cells are finally resuspended in 1/20th volume of HEPES/sucrose solution. *B. thuringiensis* competent cells (40 µl for 0.1 cm curvette and 200 µl for 0.2 cm curvette) are mixed with 5-20 µl of DNA (2-10 µg) in a prechilled electrode Gene Pulser Curvette, and exposed to the electrical current in the Gene Pulser electroporation apparatus. The parameters for the electroporation are 0.9 kV, 3 µF and R=600 for 0.1 cm curvette and 1.25 kV, 3 µF, Ω = 600 for the 0.2 cm curvette. The cells are immediately transferred to 400 µl or 1.8 ml BHIS and grown at 37°C for 4 hours at 250 rpm. During this period, the vector pSB1221 inserts into the chromosome via homologous recombination (a single cross-over) between the homologous *spoV Bt1* sequences on the bacterial chromosome and the integration vector. This results in the formation of two *spoV Bt1* genes, one on each side of the integrated DNA segment. After four hours of growth, the cells are transferred to LB agar plates containing the appropriate antibiotic. The plates are incubated overnight at 30°C and the transformants are restreaked onto fresh plates. The colonies are screened by PCR to confirm the presence of the *ermC* gene using the primers PG2 and PG4 to amplify a 0.3 kb fragment. Integration of pSB1221 into the *spoV Bt1* chromosomal locus is proven by PCR amplification of a 1261 bp produced between the upstream portions of *spoV Bt1* not included in pSB1221 integration vector using primer MS12A and the pBR322 portion of pSB1221 using primer pBR4. Results indicate that crystal encoding proteins are incorporated in the *B. thuringiensis* chromosome.

Primer	Sequence (5'-3')	SEQ ID NO.
PG2	GAAATCGGCTCAGGAAAAGG	35
PG4	AGCAAACCCGTATTCCACG	36
PBR4	GCACGATCATGCGCACCC	37

Mutant spores resulting from integration of pSB1221 did not revert to wild type and spontaneously degraded within two weeks after growth when stored in liquid or on solid bacterial media.

EXAMPLE 3: Generalized Transduction to Move the Integrated DNA to Alternative *B. thuringiensis* Hosts.

Integration occurs through homologous recombination (a double cross-over event) between DNA segments on both sides of the integrated vector in the donor strain and their homologous regions on the chromosome of the recipient strain. The donor strain containing the integrated vector is grown in 10 ml LB Ery^s plate at 30°C overnight (16-18 hours). Approximately one hundred microliters of the overnight culture are used to inoculate 10 ml LB containing 0.4% glycerol. The culture is then incubated at 30°C and 300 rpm to an OD₆₀₀ of 1-2. To infect the cells with the phage CP-51ts45, (obtained from Dr. Curtis B. Thorne, University of Massachusetts at Amherst), different amounts of the phage lysate, 1 x 10⁵ to 5 x 10⁶ plaque forming units (PFU), are added to 2 x 10⁷ cells in 3 ml Phage Assay (PA) soft agar (0.8% nutrient broth, 0.5% NaCl, 0.02% MgSO₄·7H₂O, 0.005% MnSO₄·H₂O, 0.015% CaCl₂·2H₂O, pH 6.0, 0.5% Bacto Agar) which is previously equilibrated at 50°C. The mixtures are then poured onto PA plates (PA medium containing 1.5% Bacto Agar), allowed to solidify, and incubated at 30°C for 16 hours. The top agar, which contains hundreds of plaques

in a lawn of cells, is collected in 3 to 6 ml of PA medium. The phage lysate is maintained at 16°C for 3-4 hours, centrifuged (4,000 x g, 5 minutes, 16°C), and the supernatant is filter-sterilized using a 0.45 µm filter (VWR Scientific). The phage lysate is stored at 16°C for long term storage. The titer of the phage lysate is approximately 1×10^9 to 1×10^{10} PFU/ml.

5 For generalized transduction, the G27 insecticidal gene, erythromycin marker gene and (m) *spoVBt1-8* are moved into the unmutated *spoVBt1* chromosomal locus of a *Bt kurstaki* strain which normally contains *cryIA(c)*, *IA(b)* and *IA(c)* genes. The recipient strain is grown in 10 ml LB at 30°C for 16-18 hours. Two hundred milliliters of LB are inoculated with 2-3 ml of the overnight culture and grown at 30°C and 300 rpm to an OD₆₀₀ of 1. The cells are centrifuged (7,520 x g, 4°C, 15 minutes) and resuspended in LB at a concentration of approximately 2×10^9 colony forming units (CFU) /ml (approximately 100 fold concentrated). The transduction mixture, which contains 8×10^8 recipient cells and 4.9×10^8 to 8×10^8 PFU from the phage lysate, is incubated at 37°C and 250 rpm for 30 minutes. The cell/phage suspension is spread on HA Millipore membranes (Millipore Corporation, Bedford, MA), placed on LB plates containing Ery^R, and incubated at 37°C for 3-4 hours. The membranes are then transferred to LB plates containing Ery^S and incubated at 37°C for 18-20 hours.

15 The transduced isolates are confirmed using PCR as described above and microscopic viewing of the altered spore phenotype. The amount of protein in each isolate is determined using SDS Page as well as bioassay against *T. ni* and *S. exigua*. Isolates showed production of the 135 Kdalton insecticidal crystal protein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT

(A) NAME: SANDOZ LTD
(B) STREET: Lichtstrasse 35
(C) CITY: Basel
(D) STATE: BS
(E) COUNTRY: Switzerland
(F) POSTAL CODE (ZIP): CH-4002
(G) TELEPHONE: 061-324 6428
(H) TELEFAX: 061-322 7532
(I) TELEX: 965-05055

(A) NAME: Sandoz Patent GMBH
(B) STREET: Humboldtstrasse 3
(C) CITY: Loerrach
(E) COUNTRY: Germany
(F) POSTAL CODE (ZIP): D-7850

(A) NAME: Sandoz Erfindungen Verwaltungsgesellschaft MBH
(B) STREET: Brunner Strasse
(C) CITY: Vienna
(E) COUNTRY: Austria
(F) POSTAL CODE (ZIP): A-1230

(ii) TITLE OF INVENTION: BACILLUS THURINGIENSIS SPORULATION GENE

(iii) NUMBER OF SEQUENCES: 37

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1662 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 474..1427

(D) OTHER INFORMATION: /codon_start= 474

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

5 CAGGTGAAAT GAAATCTTCG TTACGAAGTG TACGGTCTGG TTGAATAGAT ATCTCCATAT 60
 TTTTCAATGG ATTAGGAATG TTTAGAAAAT GATGCATTCT ATTTAGTACA ATAAATACAC 120
 10 GATGCATCGT TTTTCTGAG TAATGTCGAT TCGTTTTTAAA TCGGAAAAGT AATCTTCGTA 180
 GTCTTTTGTA CAAAGTGTAG CCCATATATT ACTGGAAGGG AGCTTTTGT TTTTCTTAA 240
 CCAATGTCCG AAGTCTTCAA CGTCATAAAC ATAACGTTTA ATAGTTGAGG GTTTTCGGCC 300
 15 TTTATTCAAT AAAAAAATAG AAAAGGCTTG TATTGTATCA TGGAATTCCG TTGTCTCCAT 360
 AGTCCCACCA CCTTAATTAT TTCTTATATT ATAGCAAAC TTTCTGAAAA TAGGCATTTA 420
 CAAGGGGGAC AGGAATAATA ATATTTGGTG AGTGATAAA ATGAGGTGAT TGT ATG 476
 Met
 1
 20
 GAA CAA TCG ATG CGA AAG AAA AAC AAT CAA ATT AAT ATT GTG TTA 524
 Glu Gln Ser Met Arg Lys Lys Asn Asn Asn Gln Ile Asn Ile Val Leu
 5 10 15
 25 AAC CAT CGA AAG AAA ATT TCT TTG CCA GCC GCA GAA AAT AAA ACG GTA 572
 Asn His Arg Lys Lys Ile Ser Leu Pro Ala Ala Glu Asn Lys Thr Val
 20 25 30
 30 ATT TCA AAT GAA ACT GCA ATT AAA CAT GAA ATG CTG CAG AGA ATT GAA 620
 Ile Ser Asn Glu Thr Ala Ile Lys His Glu Met Leu Gln Arg Ile Glu
 35 40 45
 GAA GAG ATG GGG AAG CTT GTT GGG ATG GAT GAT ATA AAA AAG ATA ATA 668
 Glu Glu Met Gly Lys Leu Val Gly Met Asp Asp Ile Lys Lys Ile Ile
 50 55 60 65
 35 AAA GAA ATA TAT GCT TGG ATT TAT GTG AAT AAA AAA AGA CAA GAG AAG 716
 Lys Glu Ile Tyr Ala Trp Ile Tyr Val Asn Lys Lys Arg Gln Glu Lys
 70 75 80
 40 GGA TTG AAG TCA GAG AAG CAA GTA CTT CAT ATG CTG TTT AAA GGG AAT 764
 Gly Leu Lys Ser Glu Lys Gln Val Leu His Met Leu Phe Lys Gly Asn
 85 90 95
 CCA GGT ACA GGG AAG ACA ACT GTT GCT AGA ATG ATA GGG AAA TTG CTG 812
 Pro Gly Thr Gly Lys Thr Thr Val Ala Arg Met Ile Gly Lys Leu Leu
 100 105 110
 45 TTT GAG ATG AAT ATT CTA TCG AAA GGC CAC TTG GTT GAA GCT GAA CGT 860
 Phe Glu Met Asn Ile Leu Ser Lys Gly His Leu Val Glu Ala Glu Arg
 115 120 125
 50 GCT GAT CTT GTA GGA GAG TAC ATC GGC CAT ACA GCT CAA AAA ACA AGA 908
 Ala Asp Leu Val Gly Glu Tyr Ile Gly His Thr Ala Gln Lys Thr Arg
 130 135 140 145
 GAC TTA ATA AAA AAA GCA ATG GGA GGT ATT TTG TTT ATT GAT GAG GCG 956
 Asp Leu Ile Lys Lys Ala Met Gly Gly Ile Leu Phe Ile Asp Glu Ala
 150 155 160
 55

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TAT TCT TTA GCT CGA GGA GGA GAG AAG GAC TTT GGA AAA GAA GCA ATT 1004
 Tyr Ser Leu Ala Arg Gly Gly Glu Lys Asp Phe Gly Lys Glu Ala Ile
 165 170 175
 5 GAT ACG CTT GTA AAA CAT ATG GAA GAT AAA CAA CAT GGT TTT GTA TTG 1052
 Asp Thr Leu Val Lys His Met Glu Asp Lys Gln His Gly Phe Val Leu
 180 185 190
 ATT TTA GCT GGA TAT TCA AGA GAG ATG AAT CAC TTT CTT TCA TTA AAT 1100
 Ile Leu Ala Gly Tyr Ser Arg Glu Met Asn His Phe Leu Ser Leu Asn
 195 200 205
 10 CCA GGG CTG CAA TCT CGT TTT CCA TTT ATT ATT GAA TTT GCG GAT TAC 1148
 Pro Gly Leu Gln Ser Arg Phe Pro Phe Ile Ile Glu Phe Ala Asp Tyr
 210 215 220 225
 15 TCG GTA AAT CAG TTG TTG GAA ATT GGG AAA AGA ATG TAT GAA GAT CGT 1196
 Ser Val Asn Gln Leu Leu Glu Ile Gly Lys Arg Met Tyr Glu Asp Arg
 230 235 240
 GAA TAT CAG TTA TCG AAA GAG GCT GAA TGG AAA TTT AGG GAT CAT TTA 1244
 Glu Tyr Gln Leu Ser Lys Glu Ala Glu Trp Lys Phe Arg Asp His Leu
 245 250 255
 20 CAT GCT GTA AAG TAT TCG TCG CAA ATT ACA TCG TTT AGT AAT GGG CGG 1292
 His Ala Val Lys Tyr Ser Ser Gln Ile Thr Ser Phe Ser Asn Gly Arg
 260 265 270
 25 TAT GTA CCG AAT ATT GTT GAA AAA TCA ATT CGT ACA CAG GCG ATG CGG 1340
 Tyr Val Arg Asn Ile Val Glu Lys Ser Ile Arg Thr Gln Ala Met Arg
 275 280 285
 30 TTG TTG CAA GAA GAT GCC TAT GAT AAA AAT GAT TTA ATT GGA ATA TCG 1388
 Leu Leu Gln Glu Asp Ala Tyr Asp Lys Asn Asp Leu Ile Gly Ile Ser
 290 295 300 305
 AGT ATG GAT TTG ATG CTC GAA GAG GAG ACG CAC AGT ACA TAAACTGTGC 1437
 Ser Met Asp Leu Met Leu Glu Glu Glu Thr His Ser Thr
 310 315
 35 GTCGATTTTT GTGTATAAGT TCGTTTACTC TTTTCTTGGT GTACTTCATG 1497
 GAAGTGTTC ATTTTAGCGC TCTTTTCGTG TGCTGAATTA GGATCGTGTC CAAATTGATT 1557
 40 TACTGAGCTT TTTGAGCTC CTTTATTAAC GTGGTTTGTC ATTTGTATTC ACCTCACTTT 1617
 AAAAATTAGT ATAAACATTA TATAAAGAAA AAATCGTTAG AAAGA 1662

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 318 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Gln Ser Met Arg Lys Lys Asn Asn Asn Gln Ile Asn Ile Val
 1 5 10 15

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Leu Asn His Arg Lys Lys Ile Ser Leu Pro Ala Ala Glu Asn Lys Thr
 20 25 30
 Val Ile Ser Asn Glu Thr Ala Ile Lys His Glu Met Leu Gln Arg Ile
 5 35 40 45
 Glu Glu Glu Met Gly Lys Leu Val Gly Met Asp Asp Ile Lys Lys Ile
 50 55 60
 Ile Lys Glu Ile Tyr Ala Trp Ile Tyr Val Asn Lys Lys Arg Gln Glu
 10 65 70 75 80
 Lys Gly Leu Lys Ser Glu Lys Gln Val Leu His Met Leu Phe Lys Gly
 85 90 95
 Asn Pro Gly Thr Gly Lys Thr Thr Val Ala Arg Met Ile Gly Lys Leu
 15 100 105 110
 Leu Phe Glu Met Asn Ile Leu Ser Lys Gly His Leu Val Glu Ala Glu
 115 120 125
 Arg Ala Asp Leu Val Gly Glu Tyr Ile Gly His Thr Ala Gln Lys Thr
 20 130 135 140
 Arg Asp Leu Ile Lys Lys Ala Met Gly Gly Ile Leu Phe Ile Asp Glu
 145 150 155 160
 Ala Tyr Ser Leu Ala Arg Gly Gly Glu Lys Asp Phe Gly Lys Glu Ala
 25 165 170 175
 Ile Asp Thr Leu Val Lys His Met Glu Asp Lys Gln His Gly Phe Val
 30 180 185 190
 Leu Ile Leu Ala Gly Tyr Ser Arg Glu Met Asn His Phe Leu Ser Leu
 195 200 205
 Asn Pro Gly Leu Gln Ser Arg Phe Pro Phe Ile Ile Glu Phe Ala Asp
 35 210 215 220
 Tyr Ser Val Asn Gln Leu Leu Glu Ile Gly Lys Arg Met Tyr Glu Asp
 225 230 235 240
 Arg Glu Tyr Gln Leu Ser Lys Glu Ala Glu Trp Lys Phe Arg Asp His
 40 245 250 255
 Leu His Ala Val Lys Tyr Ser Ser Gln Ile Thr Ser Phe Ser Asn Gly
 260 265 270
 Arg Tyr Val Arg Asn Ile Val Glu Lys Ser Ile Arg Thr Gln Ala Met
 45 275 280 285
 Arg Leu Leu Gln Glu Asp Ala Tyr Asp Lys Asn Asp Leu Ile Gly Ile
 290 295 300
 Ser Ser Met Asp Leu Met Leu Glu Glu Glu Thr His Ser Thr
 50 305 310 315

55

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGCTTTCGCT ACCTGGAGAG ACGCGCCCGC

30

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCAGACCAAC TGGTAATGGT AGCGACCGGC

30

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 19 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAGAGATGTC ACCGTCAAG

19

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 18 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCTGTACCT GGATTCCC

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGAATCCAG GTACAGGG

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCATCCCAAC AAGCTTCCC

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGGAAGCTTG TTGGGATGG

19

5 (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

15

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

20

CCTGTCCCC TTGTAAATGC

20

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

30

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCATTACAA GGGGGACAGG

20

(2) INFORMATION FOR SEQ ID NO:12:

40

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

50

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGCCGTCTAC TTACAAGCAG C

21

55

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGTGGTGGGA CTATGGAG

18

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTCCATAGTC CCACCACC

18

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGAGGAGGAG AGAAGGAC

18

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTCCTTCTCT CCTCCTC

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CACGATGCAT CG

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CACGATGCAT CG

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGAAAGAGGC TGAATGG

17

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGGCGGTATG TACGG

15

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CCGTACATAC CGCCC

15

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCATCAAATC CATACTCGAT ATTCC

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CGAGTATGGA TTTGATGCTC G

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGACACGATC CTAATTCAGC

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GCTGAATTAG GATCGTGTCC

20

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GATGTGATTG TAAGGAACAA TCGAAGCGAT AGAAAAAC

38

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GATCTTGAT GAGAGTAAAT CGGCCATACA GC

32

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(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GCTGTATGGC CGATTACTC TCATACAAGC TC

32

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CTATACAGCA TGTTAATGAT CCC

23

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

AGCTTGCGGC CGCGTCGACC CCGGGCCATG GGGGCCCG

38

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AATTCGGGCC CCCATGGCCC GGGGTCGACG CGGCCGCA

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GCGAAAGAAA AACACAATC

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GGAACGCTAC ATACTAGTGA TAGAGTAG

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GCTTGTACAC CGCAACTGTT TTCGCATG

28

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GAAATCGGCT CAGGAAAAGG

20

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

AGCAAACCCG TATTCACG

19

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCACGATCAT GCGCACCC

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Claim

1. An isolated spoV DNA sequence selected from the group consisting of
 - i) a *spoVBt1* gene having the nucleotide sequence shown in SEQ ID NO.1;
 - ii) a nucleotide sequence encoding a *Bacillus thuringiensis* sporulation protein as depicted in SEQ ID NO.2;
 - iii) a nucleotide sequence encoding a *Bacillus thuringiensis* sporulation protein substantially similar to the protein depicted in SEQ ID NO.2;
 - iv) a nucleotide sequence which hybridizes to a complementary strand of a sequence of i), ii) or iii) under stringent hybridization conditions and
 - v) a truncated nucleotide sequence of i), ii), iii) or iv) above wherein said truncated sequence includes at least 300 nucleotides.
2. A DNA segment comprising the spoV DNA sequence according to claim 1 and DNA sequences encoding at least one insecticidal crystal protein.
3. The DNA segment according to claim 2 further comprising an origin of replication from a gram negative bacterium and a selectable marker.
4. The DNA segment according to claim 2 wherein the DNA sequence encodes a hybrid toxin protein wherein said hybrid protein comprises domains of two or three different crystal encoding toxins.
5. A method of preparing a transformed *Bacillus thuringiensis* host expressing one to three exogenous crystal protein encoding genes comprising
 - a) obtaining a DNA segment according to claim 2;
 - b) introducing said segment into a *Bacillus thuringiensis* host;
 - c) allowing homologous recombination between the DNA segment and a substantially homologous nucleotide fragment of a sporulation gene in the host *Bacillus thuringiensis* chromosome wherein the DNA segment is stably integrated into the *Bacillus thuringiensis* host chromosome; and
 - d) isolating stably transformed *Bacillus thuringiensis* transformants
 wherein said stable transformed *Bacillus thuringiensis* is capable of producing the exogenous crystal protein.
6. An insecticidal composition comprising an insecticidally effective amount of the *Bacillus thuringiensis* host prepared according to claim 5.
7. An isolated mutated spoV DNA sequence selected from the group consisting of
 - i) the nucleotide sequence of SEQ ID No.1;
 - ii) a nucleotide sequence encoding a *Bacillus thuringiensis* protein as depicted in SEQ ID No.2;
 - iii) a nucleotide sequence encoding a *Bacillus thuringiensis* sporulation protein substantially similar to the protein depicted in SEQ ID No.2;
 - iv) a nucleotide sequence which hybridizes to a complementary strand of a sequence of i), ii) or iii) under stringent hybridization conditions and
 - v) a truncated nucleotide sequence of i), ii), iii) or iv) above wherein said truncated part of the nucleotide sequence includes at least 300 nucleotides.

wherein the nucleotide sequence of i), ii), iii), iv) or v) above has one or more point mutations, inserts or deletions.

8. A DNA segment comprising the mutated spoV DNA of claim 7; and one to three insecticidal crystal protein encoding genes.

9. A DNA segment comprising, the mutated spoV DNA sequence of claim 7; a crystal protein encoding gene; and an origin of replication from a gram negative bacterium.

10. A method of preparing insecticidal crystal protein producing *Bacillus thuringiensis* strains with mutant spores comprising

a) obtaining a DNA segment according to claim 9;

b) introducing said segment into a sporulating *Bacillus thuringiensis* host;

c) allowing homologous recombination to occur between the DNA segment and a substantially homologous sporulation gene fragment in the host *Bacillus thuringiensis* chromosome wherein said DNA segment including the mutated spoV DNA sequence is stably integrated into the *Bacillus thuringiensis* host chromosome; and

d) isolating stably transformed *Bacillus thuringiensis* transformants

wherein said stable transformed *Bacillus thuringiensis* produces mutant spores and is capable of producing the exogenous crystal toxin proteins.

11. An insecticidal composition comprising an insecticidally effective amount of an *Bacillus thuringiensis* strain prepared according to claim 10.

12. A method according to claim 5 further comprising transducing the transformed *Bacillus thuringiensis* host comprising;

a) exposing the *Bacillus thuringiensis* host to a transducing phage;

b) allowing the phage to replicate in the host *Bacillus thuringiensis* wherein one to three exogenous crystal protein encoding DNA sequences integrated in the host chromosome are incorporated into the phage; and

c) introducing the exogenous crystal protein encoding DNA sequences from the phage into a recipient *Bacillus thuringiensis*

wherein said introduced exogenous crystal protein encoding DNA sequences are stably incorporated into the recipient *Bacillus thuringiensis* chromosome and expressed in said recipient.

13. A method according to claim 7 further comprising transducing the transformed *Bacillus thuringiensis* host comprising;

a) exposing the *Bacillus thuringiensis* host to a transducing phage;

b) allowing the phage to replicate in the host *Bacillus thuringiensis* wherein the mutated spoV DNA sequence and one to three exogenous crystal protein encoding DNA proteins integrated in the host chromosome are incorporated into the phage; and

c) introducing the exogenous crystal toxin encoding DNA sequences from the phage into a recipient *Bacillus thuringiensis*

wherein the mutated spoV DNA sequence and said exogenous crystal protein encoding DNA sequences are stably incorporated into the recipient *Bacillus thuringiensis* chromosome and the exogenous crystal protein is expressed in said recipient wherein the recipient produces mutant spores.

14. A method of using a *Bacillus thuringiensis* chromosomal sporulation gene fragment as a locus for chromosomal

integration of a DNA segment, said segment comprising one to three insecticidal crystal protein encoding genes wherein said gene is stably integrated into the *Bacillus thuringiensis* chromosome.

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FIGURE 1

1 MEQSMRKKNN NQINIVLNHR KKISLPAAEN KTVISNETAI KHEMLQRIEE
51 EMGKLVGMDD IKKIIKEIYA WIYVKKRQE KGLKSEKQVL HMLFKGNPGT
101 GKTTVARMIG KLLFEMNLS KGHLEAERA DLVGEYIGHT AQKTRDLIKK
151 AMGGILFIDE AYSLARGGEK DFGKEAIDTL VKHMDKQHG FVLILAGYSR
201 EMNHFLSLNP GLQSRFPFII EFADYSVNQL LEIGKRMVED REYQLSKEAE
251 WKFRDHLHAV KYSSQITSFS NGRYVRNIVE KSIRTQAMRL LQEDAYDKND
301 LIGISSMDLM LEEETHST*

FIGURE 2
Sporulation gene interrupted with Tn917

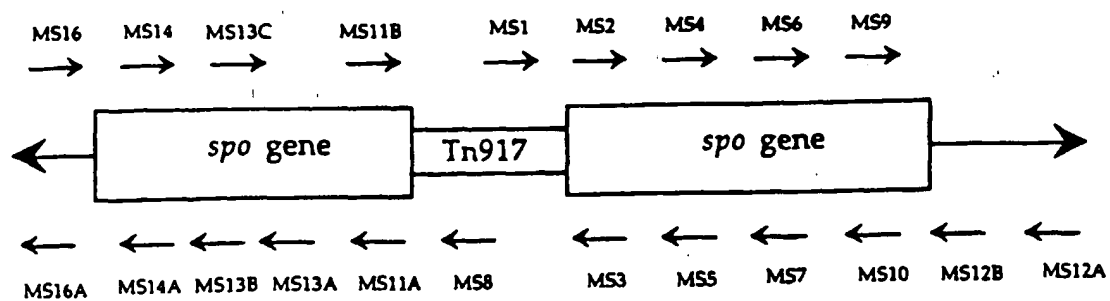
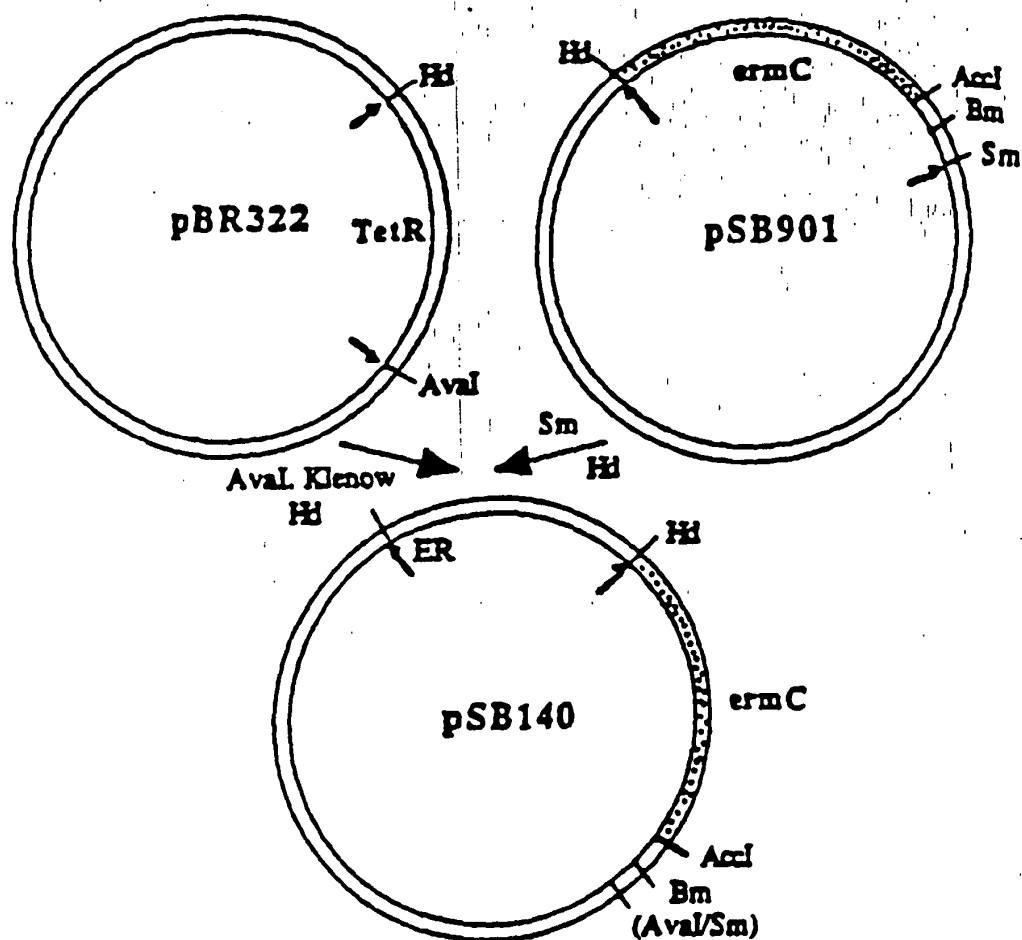


FIGURE 3



ER=EcoRI
 Kp=KpnI
 Hd=HindIII
 Bm=BamHI
 Sm=SmaI
 Sites in parenthesis were
 eliminated during cloning.

FIGURE 4

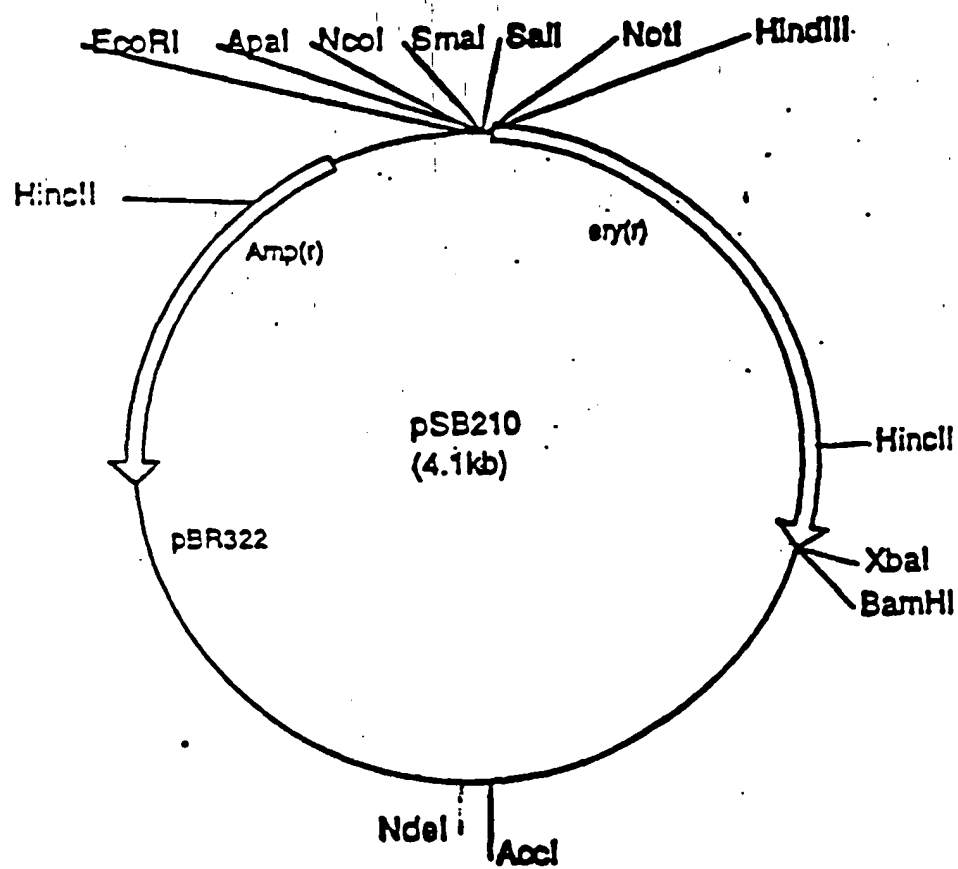


FIGURE 5

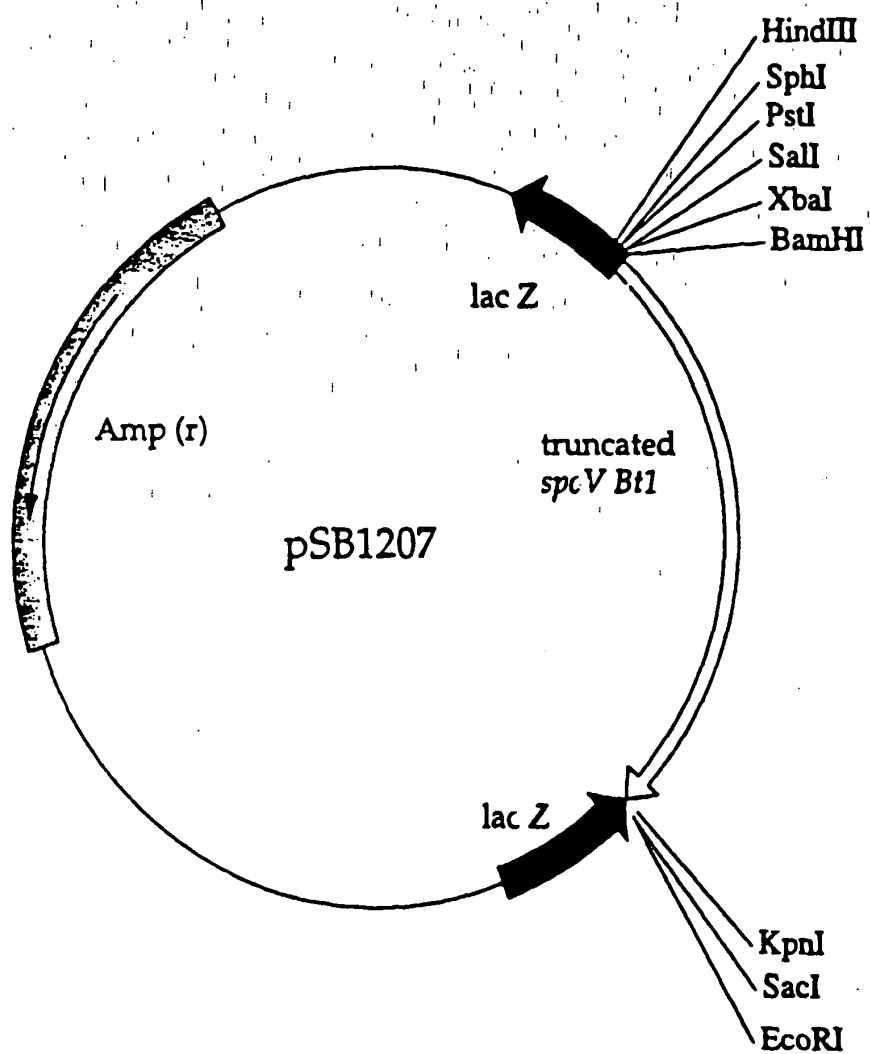


FIGURE 6

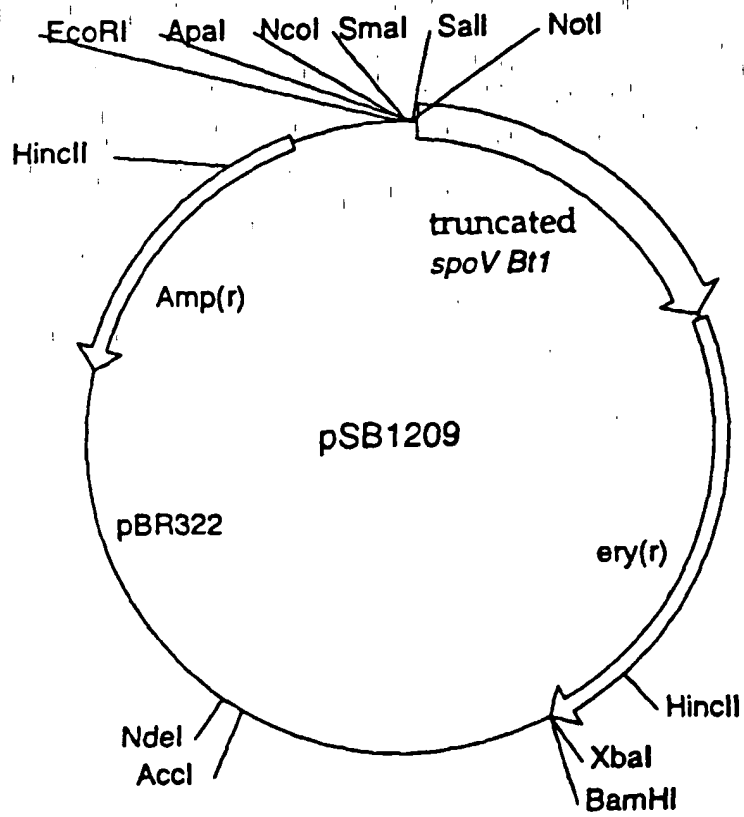


FIGURE 7

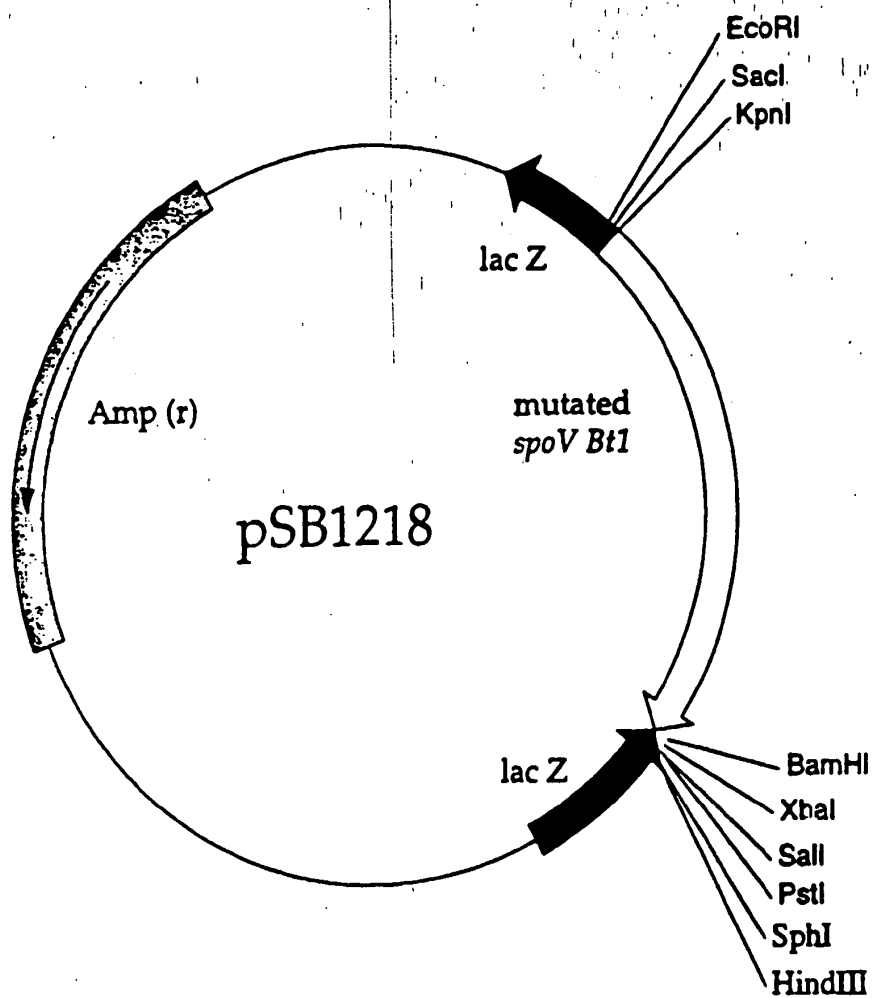


FIGURE 8

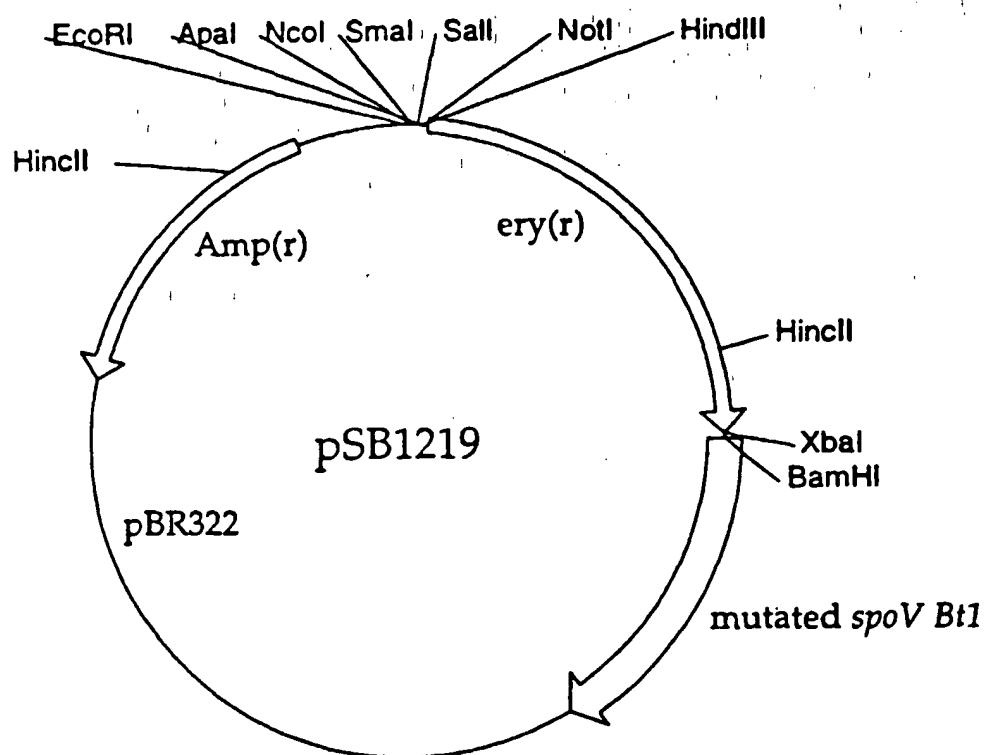


FIGURE 9

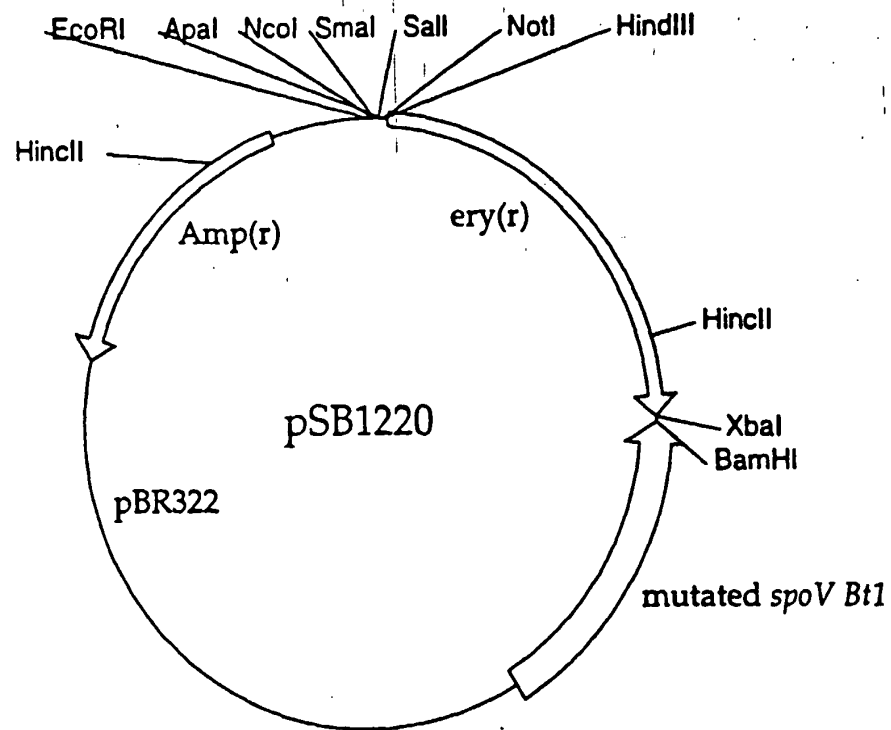


FIGURE 10

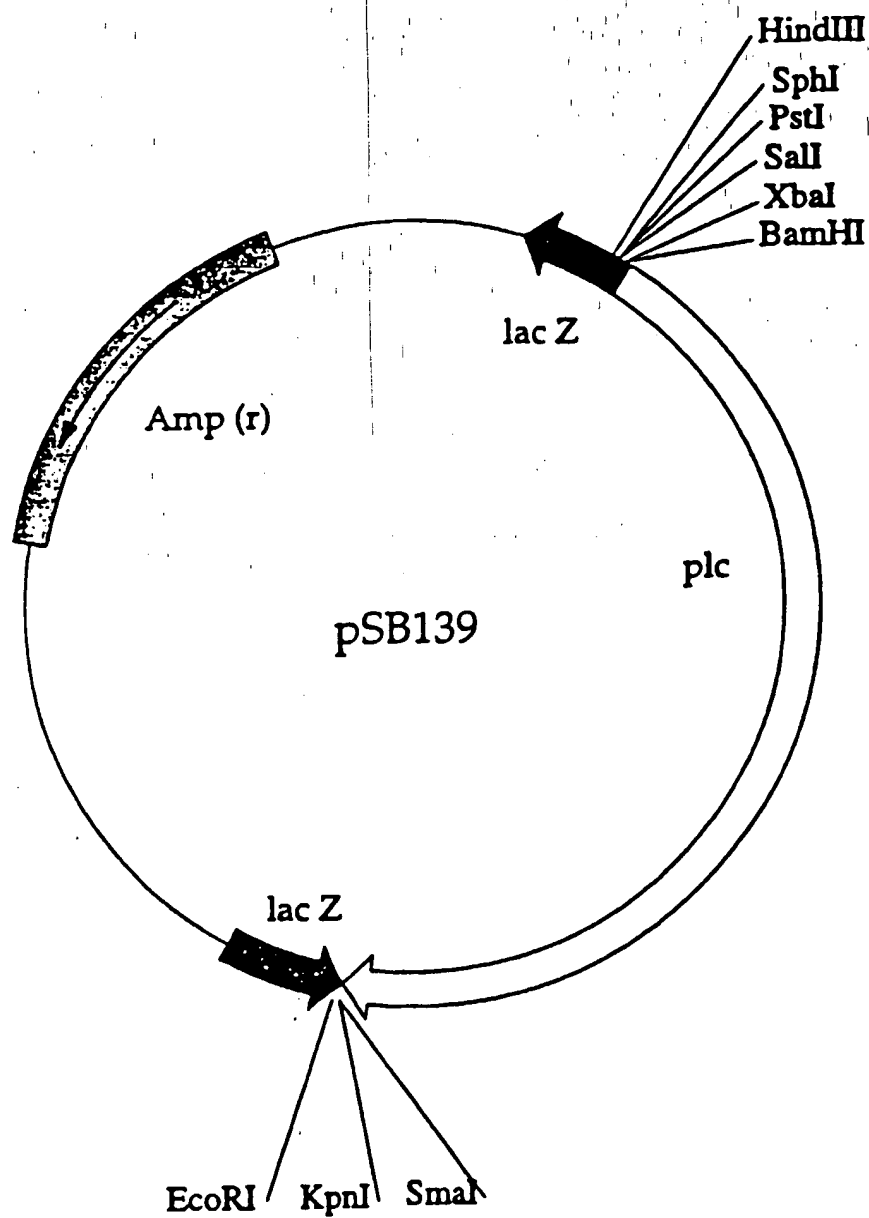


FIGURE 11

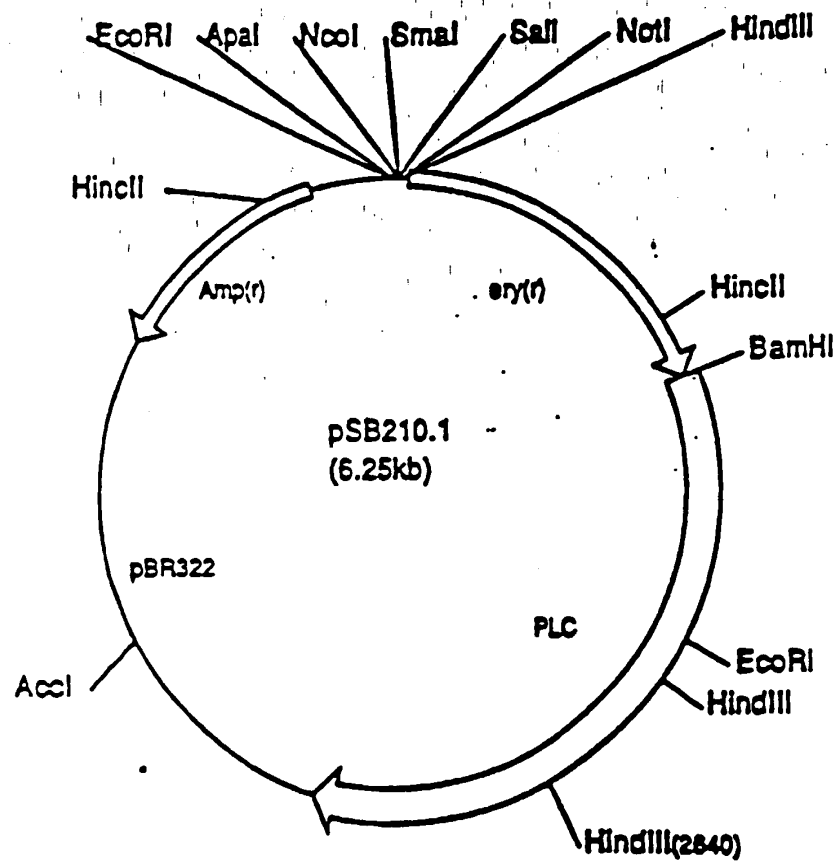


FIGURE 12

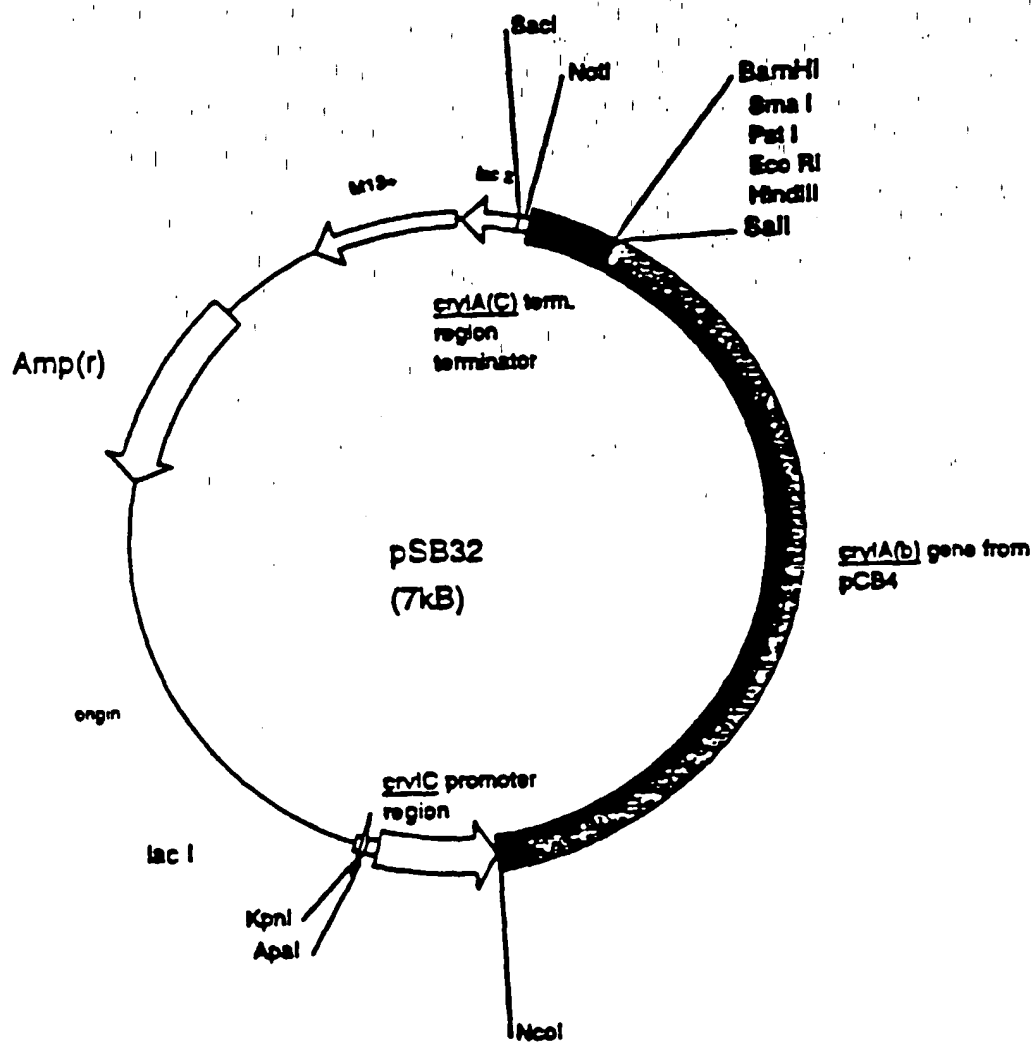


FIGURE 13

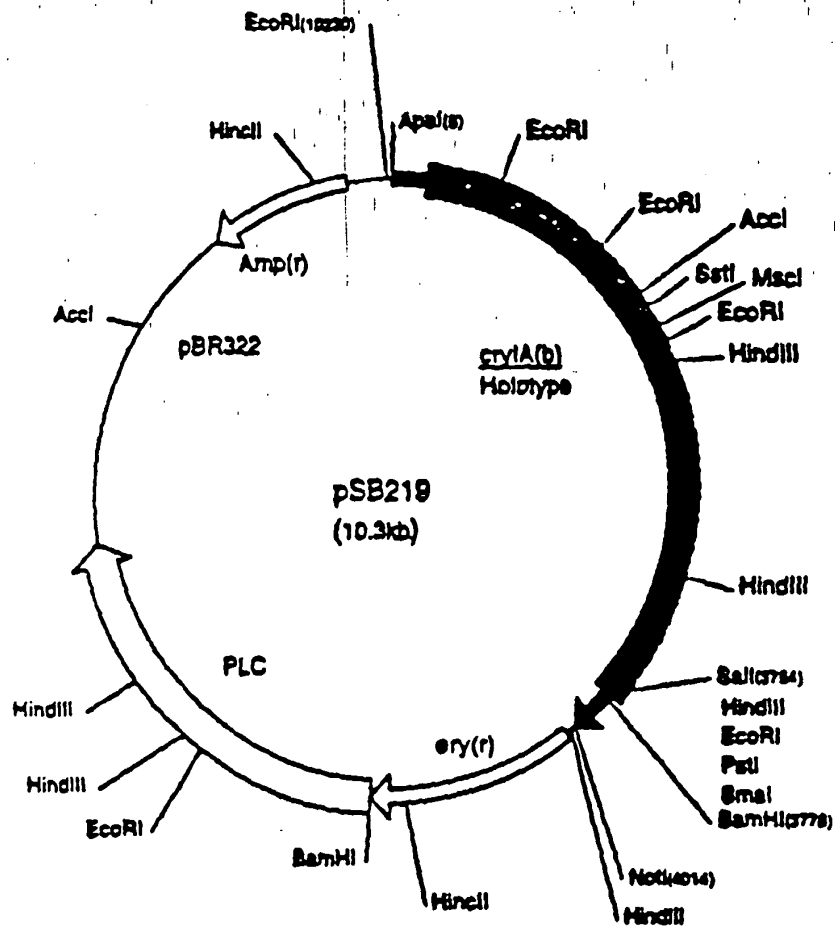


FIGURE 14

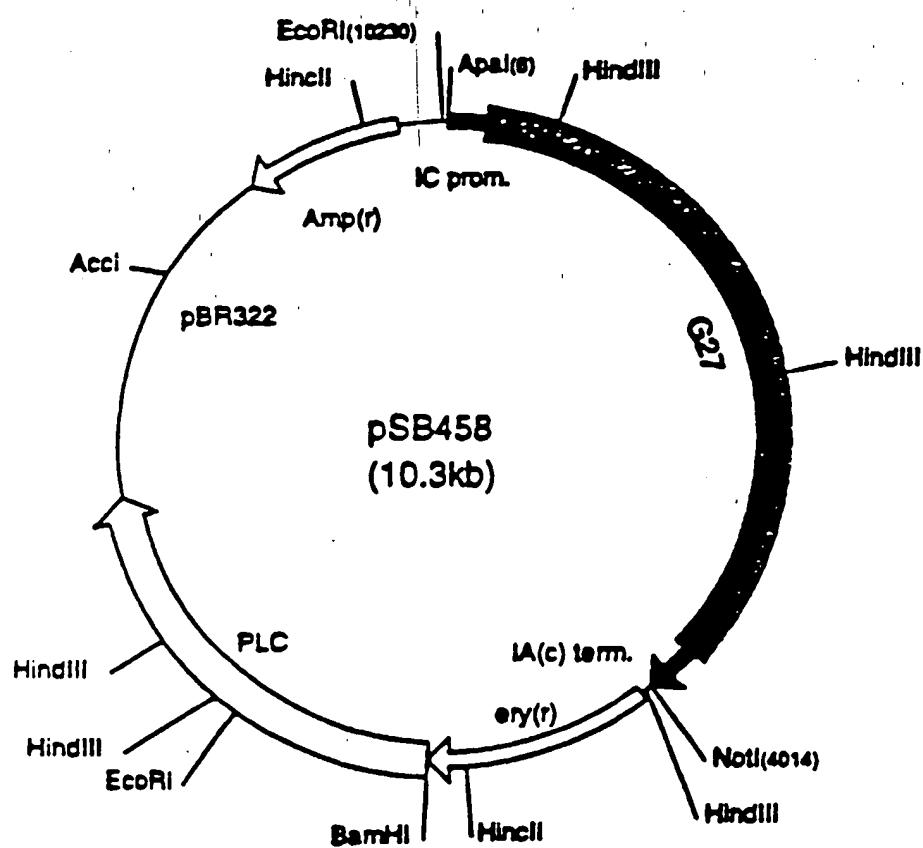
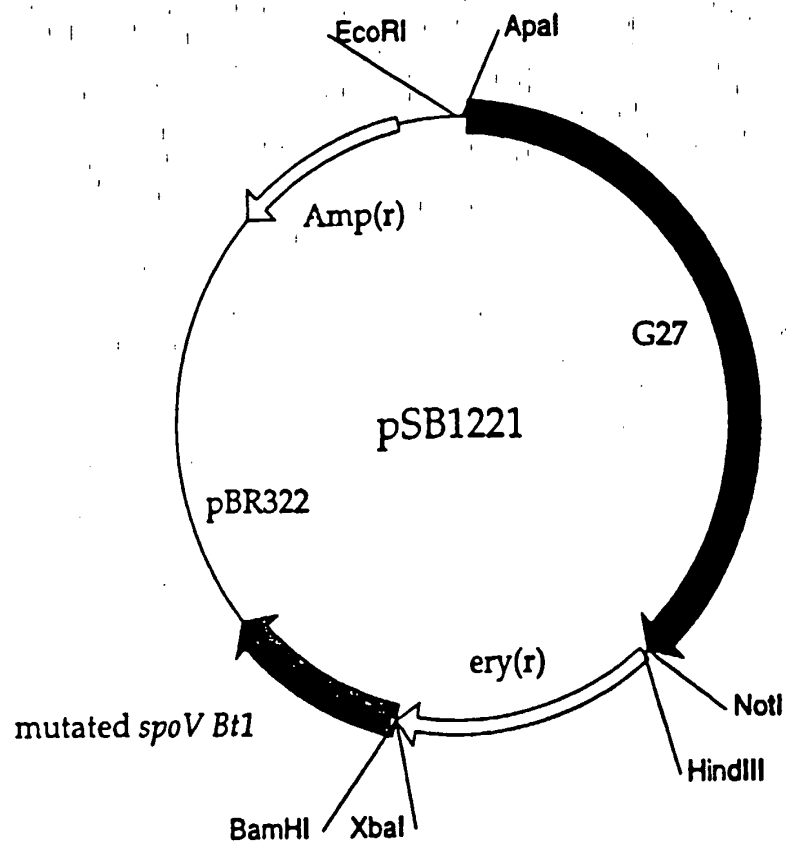
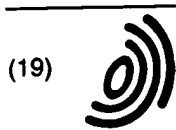


FIGURE 15





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(12)

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(54) **Bacillus thuringiensis sporulation gene**

(57) The present invention relates to a novel sporulation gene isolated from *B. thuringiensis*, and a DNA segment comprising the nucleic acid sequences encoding the gene and DNA sequences encoding crystal toxin proteins wherein said DNA segment is stably integrated into a *B. thuringiensis* host by homologous recombination.

The invention further relates to a DNA segment wherein the sporulation gene is mutated thereby rendering an oligosporogenic or asporogenic transformed *B. thuringiensis* as a result of stable integration of the DNA segment into the *Bacillus thuringiensis* chromosome by homologous recombination.

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European Patent
Office

EUROPEAN SEARCH REPORT

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D, X	FOULGER D, ERRINGTON J : "SEQUENTIAL ACTIVATION OF DUAL PROMOTERS BY DIFFERENT SIGMA FACTORS MAINTAINS spoVJ EXPRESSION DURING SUCCESSIVE DEVELOPMENTAL STAGES OF <i>Bacillus subtilis</i> " MOLECULAR MICROBIOLOGY, vol. 5, no. 6, June 1991, pages 1363-1373, XP002103043	1, 7	C12N15/32 C12N15/75 C07K14/325 C12N1/21 //C12N15/62, (C12N1/21, C12R1:19)
Y	* the whole document *	1-14	
Y	LERECLUS D ET AL: "OVERPRODUCTION OF ENCAPSULATED INSECTICIDAL CRYSTAL PROTEINS IN A <i>BACILLUS THURINGIENSIS</i> SPOOA MUTANT" BIO/TECHNOLOGY, vol. 13, no. 1, 1 January 1995, pages 67-71, XP002007696 * the whole document *	1-14	
Y	YOSHISUE H ET AL: "Cloning and characterization of a <i>Bacillus thuringiensis</i> homolog of the spoIIID gene from <i>Bacillus subtilis</i> " GENE, vol. 154, no. 1, 27 February 1995, page 23-29 XP004042497 * the whole document *	1-14	TECHNICAL FIELDS SEARCHED (Int.Cl.8) C12N C07K
A	KALMAN S, ET AL.: "ENHANCED PRODUCTION OF INSECTICIDAL PROTEINS IN <i>BACILLUS THURINGIENSIS</i> STRAINS CARRYING AN ADDITIONAL CRYSTAL PROTEIN GENE IN THEIR CHROMOSOME" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 61, no. 8, August 1995, pages 3063-3068, XP002103537 * the whole document *	12, 13	
The present search report has been drawn up for all claims			
Place of search		Date of completion of the search	Examiner
MUNICH		26 May 1999	Bilang, J
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